Genetic structure of pest polydorids (Annelida: Spionidae) infesting *Crassostrea gigas* in southern Africa: Are pests being moved with oysters?

By

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Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Zoology at Stellenbosch University

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March 2015

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Abstract

Polydorid polychaetes infest commercially important shellfish such as the oyster, *Crassostrea gigas*, and can cause financial losses to the industry. Early shipping voyages from Europe to South Africa, and the importation of oyster spat from USA, France, UK, Chile and Namibia, has most likely led to the introduction of non-native shell-boring polydorids in South Africa. Additionally, oysters are often moved between farms which may spread these pests further. The most prevalent southern African polydorids infesting farmed *C. gigas* are the indigenous *Boccardia pseudonatrix*, the introduced *Polydora hoplura* and a species tentatively identified as *Polydora ciliata*/*calcarea*. The aims of this study were therefore to 1) confirm the identity of *P. ciliata*/*calcarea* and to 2) determine the genetic structure of the three pests and compare these structures to a control for natural dispersal (*Boccardia polybranchia*) to determine if pests worms are a) being moved with oysters, b) moving between farm and wild sites or c) moving naturally between sites, facilitated by ocean currents along the southern African coast. Traditional taxonomic characters were used to identify species, and revealed that *P. ciliata*/*calcarea* morphologically closely resembles *Polydora websteri* from Japan and Australia. To confirm this identity, an 18S rRNA phylogeny of 1759 bp was constructed for *P. ciliata*/*calcarea*, *P. websteri* from Japan, Australia and USA and other morphologically similar species. The phylogeny supported the morphological data; southern African specimens differed by only 2 bp (0.1%) from Japanese and Australian *P. websteri* specimens. However, they all differed markedly (29 bp/1.6%) from *P. websteri* from near the type locality in the USA. It was therefore concluded that American specimens represent the "true" *P. websteri*, and that southern African, Japanese and Australian specimens represent a morphologically similar, but genetically distinct species, here referred to as *Polydora* cf. *websteri*. Analysis of the mtDNA Cytochrome b and nuDNA ATPsα datasets revealed that Cyt b was more sensitive in detecting genetic differentiation among populations, whereas the ATPsα marker showed a lack of phylogeographic structure. The Cyt b haplotype network constructed for *B. polybranchia* showed a high level of genetic structure between east and west coast populations, which is concordant with a documented barrier to gene-flow at Cape Point. However, genetic structure among east coast populations was discordant with all other documented barriers to gene-flow in that region. The genetic distribution of *B. polybranchia* suggests that dispersal is primarily influenced by local ocean currents. Haplotype networks for *B. pseudonatrix* show some genetic structure among farms, suggesting independent sources of infestation and localised movement between wild and farmed sites, with some

inconclusive evidence for anthropogenic movement between Kleinzee and Hamburg farms. Populations of *P. hoplura* show some genetic structure among neighbouring sites, probably due to localised dispersal of larvae, however, there is substantial evidence for the anthropogenic dispersal of this species. *Polydora* cf. *websteri* revealed a single Cyt b haplotype for all populations, providing some evidence for a single introduction from a single source population. Due to the absence of variation in this marker it is not possible to make any inferences on anthropogenic dispersal Overall, both introduced species show no evidence of genetic structure which could be attributed to anthropogenic dispersal. These results suggest that caution should be exercised with the movement of molluscs since shell-boring polydorids are likely to be moved with them.

Opsomming

Polydorid polikete infesteer kommersiële belangrike skulpvisse soos die oester, *Crassostrea gigas*, en dit kan tot finansiële skade in die industrie lei. Vroëe verskepingsritte vanaf Europa na Suid-Afrika en die invoer van oesters vanaf die VSA, Frankryk, Engeland, Chillie en Namibië het tot die invoer van indringer uitheemse skulp-borende polydorids in Suid Afrika gelei. Aanvullend tot dit word oesters tussen plase verskuif en dit versprei die peste verder. Die mees algemeenste polydorids wat geboerde *C. gigas* besmet in suidelike Afrika is die inheemse *Boccardia pseudonatrix*, die indringer spesies *Polydora hoplura* en 'n spesie wat voorlopig as *Polydora ciliata*/*calcarea* geidentifiseer is. Die doelwitte van die studie is om 1) die identiteit van *P. ciliata*/*calcarea* te bevestig en 2) om die genetiese struktuur van die drie peste te bepaal en te vergelyk met 'n kontrole vir natuurlike verspreiding (*Boccardia polybranchia*) om vas te stel a) of peste met oesters versprei word, b) of daar beweging tussen plase en wilde lokaliteite is of c) natuurlike beweging tussen lokaliteite gefasiliteer word deur seestrome langs die suidelike Afrika kuslyn. Tradisionele taksonomiese karakters was gebruik om spesies te identifiseer en het bewys dat *P. ciliata*/*calcarea* morphologies baie na aan *Polydora websteri* van Japan en Australië is. Om hierdie identifikasie te bevestig is 'n 18S rRNA filogenie van 1759 bp gekonstrueer vir *P. ciliata*/*calcarea*, *P. websteri* van Japan, Australië en die VSA en ander morfologies soortgelyke spesies. Die filogenie het die morfologiese data ondersteun, suidelike Afrikaanse eksemplare verskil slegs 2 bp (0.1%) van die Japanese en Australiese eksemplare. Hierdie groep het egter grootliks verskil (29 bp/1.6 %) van *P. websteri* wat versamel is naby die tipe lokaliteit in die VSA. Die gevolgtrekking was dat Amerikaanse eksemplare die "ware" *P. websteri* verteenwoordig en dat suidelike Afrika, Japenese en Australiese eksemplare 'n morfologiese soortgelyke, maar genetiese spesifieke spesies verteenwoordig, hier verwys na as *Polydora* cf. *websteri*. Analise van die mtDNA Cytochrome b en nuDNA ATPsα datastelle het bewys dat die Cyt b meer sensitief is om genetiese differensiasies tussen bevolkings op te spoor waar die ATPsfix merker 'n tekort van filogeografiese struktuur gewys het. Die Cyt b haplotiepe network gekonstruktueer vir *B. polybranchia* toon 'n hoë vlak van genetiese struktuur tussen oos en weskus bevolkings wat in ooreenstemming is met die gedokumenteerde hindernisse tot geenvloei by Kaappunt. Genetiese struktuur tussen die ooskusbevolkings was nie geaffekteer deur hindernisse vir geenvloei in daardie area nie. Die genetiese verspreiding van *B. polybranchia* suggereer dat verspreiding word primêr deur plaaslike see strome beïnvloed. Haplotipe netwerke vir *B. pseudonatrix* toon geringe genetiese struktuur tussen plase wat op onafhanklike bronne van

besmetting dui, en plaaslike beweging tussen wilde en geboerde lokasies met moontlike antropogeniese beweging tussen Kleinzee en Hamburg plase. Populasies van *P. hoplura* toon genetiese struktuur tussen naburige lokaliteite, waarskynlik as gevolg van gelokaliseerde verspreiding van larwas, maar daar was genoegsame bewys vir antropogeniese verspreiding van die spesies. *Polydora* cf. *websteri* het 'n enkel Cyt b haplotipe vir alle populasies bewys wat dui op ‘n enkele vestiging. As gevolg van die feit dat alle diere dieselfde haplotipe deel kon geen uitspraak gemaak word oor antropogeniese verspreiding nie. Oor die geheel, toon beide spesies geen duidelike biogeografiese genetiese struktuur nie wat moontlik kan wys op antropogeniese verspreiding. Hierdie resultate suggereer dat die beweging van skulpvisse versigtig gedoen moet word aangesien skulp-borende spesies ook saam beweeg kan word.

Acknowledgements

I would like to sincerely thank my supervisors Carol Simon and Conrad Matthee for their invaluable knowledge, guidance and patience with this project.

I would like to thank the National Research Foundation (Thuthuka Program) of South Africa for funding as well as the Department of Agriculture Forestry and Fisheries (DAFF) for the permits issued for sampling along the coast. I would also like to thank the Marine lab for their valuable comments and support during the course of this degree. Additional thanks to Quirin Snethlage, Kliffie Smit, Kevin Ruck, Simon Burton and Dave Krebser for providing me with oysters and invaluable information regarding their respective farming operations. This project would not have been possible without their contributions.

For assistance with field collections I would like to thank Andrew David, Melissa Boonzaaier, Ethan Newman, Brendan Havenga and Jonathan Jonker. Additional thanks to Sandy van Niekerk and Jaco Visser for assisting me with the identification of worms and the use of genetic programs respectively.

Finally, I would like to thank my mother Ursula Williams, father Gavin Williams and brother Declyn Williams for all the support over the last three years.

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Chapter 1

Introduction

1.1 Shell-boring polydorids and their effect on molluscs

Some of the most important pests of cultured molluscs include members of the *Polydora*-complex, commonly known as polydorids (Annelida: Spionidae). The Polydora-complex currently consists of over 161 species comprising nine genera; *Polydora* Bosc, 1802; *Dipolydora* Verril, 1879; *Pseudopolydora* Czerniavsky, 1881; *Boccardia* Carazzi, 1893; *Tripolydora* Woodwick, 1964; *Carazziella* Blake and Kudenov, 1978; *Boccardiella* Blake and Kudenov, 1978; *Polydorella* Augener, 1914 and *Amphipolydora* Blake, 1983 (Simon, 2011; Walker, 2014).

Shell-boring polydorids (natural symbionts of oysters and other molluscs in the wild) can become a problem when culturing molluscs on a commercial scale (Walker, 2014). High polydorid infestations often have a negative effect on the host shell and tissue condition (Kojima and Imajima, 1982). For example, an intensity of ten or more polydorids per shell greatly decreases the growth of abalone (Kojima and Imajma, 1982; Giribet and Wheeler, 2002). However, this may depend on the polydorid species and the size of the infested abalone, as larger individuals are usually less susceptible to the effects of infestation than smaller individuals (Simon *et al*. 2006). Some studies have shown that the flesh condition index of the Pacific oyster, *Crassostrea gigas* Thunberg is decreased by high polydorid infestation (Nel *et al*. 1996; Handley and Bergquist, 1997; Caceres-Martinez *et al*. 1998), presumably as a consequence of more energy being allocated to shell repair than to body growth and development (Handley, 1998; Simon *et al*. 2006; Sato-Okoshi *et al*. 2008). However, the negative effects of polydorid infestations are not only restricted to the decreased shell and tissue conditions of the host, as *Polydora* sp. infestations have been shown to modify the respiratory behaviour of *C. gigas* (Chambon *et al*. 2007). In extreme cases infestation may cause death, thereby further reducing the commercial output of the associated shellfish farm (Day, 1967; Lauckner, 1983; Blake, 1996).

Oysters are highly susceptible to fouling organisms since they do not bore into the substratum, leaving them exposed. Furthermore, the rugose nature of oyster (and other molluscan) shells makes the removal of fouling organisms more problematic (Wolff and Reise, 2002; Haydar and Wolff, 2011). Since pest polydorids bore into the shells of molluscs, it increases the probability that these species may be moved with these molluscs. Since South Africa has an extensive history of oyster farming, which often includes the movement of oysters; it is important to consider how this may have influenced the movement of fouling species such as shell-boring polydorids.

1.2 Oyster farming in South Africa – Historical and current, and implications for inadvertently importing and transporting pests.

Hecht and Britz (1990) summarised the early history of the culture and importation of oysters into southern Africa. The first attempts at culturing indigenous oysters in South Africa were made between 1673 and 1676. These attempts were, however, unsuccessful since none of the introduced oysters survived. Subsequently, in 1893 an attempt was made at culturing oysters of European origin, when 1000 individuals (probably *Ostrea edulis*) were imported from England and France. These oysters were introduced at Swartkops Estuary in the Eastern Cape Province, but again the introduction was unsuccessful. A further consignment of English oysters was imported the following year, and these were laid down in the mouth of the Berg River and in Saldanha Bay. As with the previous attempts, this was also unsuccessful. In all instances it was not mentioned how long the animals survived before dying.

After years of experimental trials on the sustainability and profitability of imported oysters, the South African oyster industry is now based entirely on the Pacific oyster, *C. gigas*, imported as spat. The local industry does not have the required hatchery facilities to culture the larvae (Hecht and Britz, 1990; Haupt *et al*. 2010a). *Crassostrea gigas* spat was first imported to the Knysna Estuary in 1973 where oyster culture continued for many decades (Hecht and Britz, 1990). Since 2001, production has significantly decreased and has rather been concentrated in Saldanha Bay and Port Elizabeth (Haupt *et al.* 2010a). At the time of commencement of the current study, oysters were cultured in Swakopmund and Walvis Bay in Namibia and also at, Kleinzee (Northern Cape), Paternoster and Saldanha Bay (Western Cape) and Port Elizabeth and Hamburg (Eastern Cape) in South Africa. The oyster farm in Paternoster closed down in 2012.

Currently the South African oyster industry relies on a complex system of importing spat and the translocations of juvenile and mature oysters between nurseries and local farms (Haupt *et al.* 2010a; Haupt *et al.* 2012). Haupt *et al.* (2010a) reported that oyster nurseries in Walvis Bay, Kleinzee, Paternoster and Jeffrey's Bay import *C. gigas* spat from Chile, France and the United Kingdom, after which the spats are kept in upwelling facilities for approximately two months. When oysters reach approximately 20-25 mm, they are suspended in plastic mesh cages in the upper water column in ponds (Kleinzee and Paternoster), or in the open sea (Walvis Bay). After this, oysters are cleaned and cultured until they reach the required size for transport to grow-out farms in various regions along the coast. It is unknown to what extent oysters are cleaned before

the transport to the grow-out farms. In some cases, juvenile oysters are translocated to a different farm where conditions may be better suited for growth, and returned to the original site when a suitable size is reached (Haupt *et al*. 2012).

The continuous importation of oyster spat from various countries, and the movement of oysters between farms may lead to the introduction and spread of indigenous and non-indigenous pest species such as shell-boring polydorids, especially if there are no proper precautions or thorough screening for these species (Wolff and Reise, 2002; Haupt *et al.* 2010b). Moreno *et al*. (2006) suggested that the on-going movement of oysters has resulted in the secondary spread of shell-boring polychaetes in Chile. In addition to this, it was also reported that some polychaete species introduced to Chile via aquaculture were able to infest nearby populations of native host species, which further promotes the spread of the introduced worms.

In addition to potential introductions and secondary spread of shell-boring species via aquaculture, the location of local grow-out farms may play a role in the spread of shell-boring species. Oysters at Swakopmund, Walvis Bay, Kleinzee and Paternoster are farmed onshore in ponds, while Saldanha Bay, Port Elizabeth and Hamburg farms are marine based (located offshore) (Haupt *et al*. 2010a; Smit and Krebser (oyster farmers from Paternoster and Hamburg), pers. comm.). At off-shore farms there would presumably be less larval retention relative to onshore farms since larvae may be more easily dispersed away from the source population due to their exposure to ocean currents. However, onshore farmers may more easily implement precautionary measures (regular cleaning of ponds and oysters) to minimise infestation, that may prevent proliferation of these pests. In contrast, in an offshore environment, farmers cannot control the exposure of the oysters to planktotrophic larvae of fouling species (such as shell-boring polydorids) that may be present in the water column.

Around the world, oyster transport has become increasingly important in the translocation and spread of fouling species (Naylor *et al.* 2001; Wasson *et al*. 2001; Wolff, 2005; Haupt *et al.* 2010a; Haydar and Wolff, 2011 and references therein). This is a consequence of the large quantities of oysters shipped, the long history of the trade (Wolff and Reise, 2002; but see Hecht and Britz, 1990), and the increase in frequency of oyster transport in recent years (Haupt *et al*. 2010b; Haydar and Wolff, 2011). Examples of some species that may have been introduced to South Africa with oysters include: the black sea urchin, *Tetrapygus niger*; the European flat oyster, *O. edulis*; Montagu's crab, *Xantho incisus*; and the brachiopod, *Discinisca tenius* (Haupt

et al. 2010b). However, it is not clear whether these species have been further dispersed from the point of introduction, or whether it is likely that there have been multiple introductions at various sites along the coast.

Examples of polydorids that have been introduced into foreign countries include *Polydora websteri* Hartman, 1943 which was probably inadvertently introduced to Hawaii with oysters from Kaneohe Bay or oyster spat from the USA (Bailey-Brock and Ringwood, 1982); *Boccardia proboscidea* Hartman, 1940 which was possibly introduced to Hawaii with *O. edulis* from Maine (Bailey-Brock, 2000); *Polydora rickettsi* Woodwick, 1961 possibly introduced to Chile with *C. gigas* (Servicio Nacional de Pesca, 1999 in Moreno *et al.* 2006) and *Polydora uncinata* Sato-Okoshi, 1998 transported to Chile possibly with abalone brood stock from Japan (Radashevsky and Olivares, 2005). Shell-boring polydorids may also be transported in the packaging with the aquaculture species. An example is *Polydora nuchalis* Woodwick, 1953 that was probably transported to Hawaii with shipments of shrimp from western Mexico (Bailey-Brock, 1990). In some instances, introduced polydorids have caused significant damage and changes to local ecosystems in their introduced range. For example, in Argentina *B. proboscidea* was recorded in very high densities at a nutrient rich sewage-affected area where it displaced the ecosystem engineering mussel *Brachidontes rodriguezii* ultimately affecting the intertidal benthic community structure (Jaubet *et al.* 2011).

Although the transport of oysters is one of the most important vectors for the introduction and spread shell-boring polydorids in South Africa, there are other vectors such as ship ballast and hulls that have most likely played an important role in the introduction and possibly the spread of non-indigenous species.

1.3 Ship ballast and hull-fouling as vectors for the dispersal of fouling species in South Africa – A historical perspective

Lacour-Gayet (1997) documented the history of early shipping voyages to the Cape of Good Hope, South Africa. Bartholomew Diaz from Portugal first discovered the Cape of Good Hope in 1488. In the same year he rounded the Cape and sailed up the east coast of Africa, to some sixty miles north of what is now known as Port Elizabeth. His arrival there was followed by Vasco da

Gama, also from Portugal, in 1497. He too rounded the Cape, where he continued north-east until he reached the east coast of India. A French merchant vessel arrived there in 1527, and in 1580 Francis Drake from England. This was followed by the arrival of Cornelis de Houtman from the Netherlands in 1595. Between 1611 and 1621, as many as 117 ships had sailed to the Cape from the Netherlands, and an additional 461 the following thirty years. Jan Van Riebeeck arrived at the Cape in 1652, followed by the French Huguenots in 1688. This is not a complete account of early voyages (up until the late 17th century) to South Africa, but it serves to illustrate that marine organisms (including shell-borers) that are native to Europe may have been introduced to South Africa as early as the late 15th century.

Griffiths *et al.* (2009) discussed various vectors that may have contributed to the introduction of marine species into South Africa over time. Early wooden shipping vessels such as those that arrived in the late 15th century, hosted various specialised wood-boring species such as ship-worms (i.e. bivalve molluscs of the family *Teredinidae*), gribbles (i.e. isopods of the genus *Limnoria*) and amphipods of the family *Cheluridae*. These species were known to damage shipping vessels, which in turn increased the probability of sinking at sites where the ships docked. In addition to this, wooden vessels were ideal habitats for a large variety of sessile fouling species including tubeworms, hydroids, bryozoans, ascidians, barnacles and bivalves, providing habitats for smaller species such as amphipods, isopods and polychaetes. Furthermore, shell-boring polychaete worms may have also burrowed into the shells of the fouling molluscs and barnacles, further increasing the likelihood of introduction at recipient regions. These early ships travelled slowly, used solid ballast and had relatively long harbour residence times, which potentially increased the risk of foreign introductions (Haydar, 2010). However, these introductions would most likely be limited to marine invertebrates transported as hull-foulers or on dry ballast.

More modern steel shipping vessels also carry fouling species, however, the numbers and types of fouling species transported have changed since these ships travel faster, are generally larger and are sometimes painted with anti-fouling paint specifically designed to decrease the amount of fouling organisms on the ship (Griffiths *et al.* 2009). In addition to this, major developments have been made in terms of shipping harbours and other marine industries, e.g. coal and iron imports of Saldanha Bay and Richards Bay opened up additional foreign trade routes, and the

development of the deep-water harbour at Coega (Eastern Cape) may also open up another point of introduction for marine species (Griffiths *et al.* 2009).

Ballast water was used instead of dry ballast from the late 1870's onward (Carlton, 1987). Millions of tons of ballast water are now transported around the globe annually (Carlton and Gellar, 1993). Some studies have identified ballast water as a serious vector for introductions, especially for holoplankton (species that are planktonic their entire life) and species that have a planktonic larval phase (Carlton and Gellar, 1993; Hallegraeff, 1998; Wonham *et al.* 2001). Carlton and Gellar (1993) described ballast water as a phyletic and non-selective transport vector, but did indicate that certain taxa are more prone to transportation, among them polychaete annelids. Furthermore, ballast was generally loaded in shallow port areas where large amounts of sediment which could potentially support a significant number of infaunal species, could be taken up and translocated to recipient regions (Hewitt *et al.* 2009).

As such, ship ballast, hull-fouling and aquaculture imports have probably played a significant role in the dispersal of native and introduced shell-boring polydorids in South African waters. In fact, Mead *et al* (2011b) indicated that the three most important vectors for historical introductions of polychaete worms is ship fouling, ballast water and mariculture, with the latter playing the least significant role. However, the importation and movement of molluscs (including oysters) has most likely also led to the introduction and spread of shell-boring polydorids in South Africa (Mead *et al.* 2011b; Simon *et al.* 2009). Two major pests of aquaculture in South Africa are *Polydora hoplura* Claparéde, 1870 (first recorded in Naples, Italy) and *Boccardia proboscidea* first recorded in California, USA). Neither of these species is native to South Africa and it is not clear how they were introduced into the region (Simon *et al.* 2009; Haupt *et al.* 2010b). However, given that most of the early voyages and oyster introduction to South Africa were made from European countries, many within the distribution range of *P. hoplura*, it is possible that this species was introduced from there (Blake and Kudenov, 1978; Walker, 2011). The distribution and genetic structure of *B. proboscidea* suggests a recent introduction, possibly facilitated by aquaculture imports (Simon *et al.* 2009), or ballast water since this species has previously been shown to occur in sand flats in California, USA (Johnson, 1970), Argentina (Jaubet *et al.* 2011), and South Africa (pers. obs.), silt in Australia (Walker, 2014) and may occur in these habitats elsewhere in the world.

1.4 Shell-boring polydorids in southern Africa

In southern Africa, the *Polydora*-complex is represented by five genera; *Boccardia*, *Boccardiella*, *Dipolydora*, *Polydora* and *Pseudopolydora* (Day, 1967; Simon, 2009; Simon, 2011; Simon *et al*. 2010). Day (1967) listed five species within these genera as borers; *Dipolydora capensis* (Day 1955) recorded in high densities on abalone, *Polydora maculata* Day 1963 recorded only on the shells of hermit crabs, *Boccardia pseudonatrix* Day 1961 and *Polydora hoplura* Claparéde 1869 which were recorded boring into rock and lithothamnion respectively, while *Polydora ciliata* Johnston 1838 was recorded boring into both (Day, 1967).

Polydorids infesting farmed and wild abalone (*Haliotis midae*) in South Africa have been well documented. Abalone collected in 2004 from farms on the east, south and west coasts were infested by a *Boccardia* sp., which was later identified as *Boccardia proboscidea* (Simon *et al*. 2006, 2009, 2010). *Boccardia polybranchia* (Haswell 1885) was recorded on wild *H. midae* from Mossel Bay and Grootbank (Simon *et al*. 2009). In subsequent years, *Pseudopolydora dayii* (Simon, 2009), *Dipolydora armata* (Langerhans, 1880), and *Dipolydora normalis* (Day, 1957) were recorded on farmed abalone from Haga Haga (Simon, 2011), and *B. proboscidea* was found in sediment at a nutrient rich outflow at the Gansbaai abalone farm (David and Simon, 2014). Furthermore, various *Boccardia* (*B. proboscidea*, *B. pseudonatrix*), *Dipolydora* (*D. capensis*, *D. normalis*, *Dipolydora keulderae* (Simon, 2011), *Dipolydora* sp. 1) and *P. hoplura* and *Ps. dayii* were recorded on abalone from 14 farms along the South African coast (Boonzaaier *et al.* 2014). In addition, an extensive list of *Polydora* (*Polydora dintwanyana*, *P. hoplura*) and *Dipolydora* (*D. armata*, *D. capensis*, *Dipolydora* cf. *capensis*, *Dipolydora* cf. *giardi*, *D. keulderae*, *D. normalis*, *Dipolydora caeca* (Oersted 1843), *Dipolydora* sp. 1,2,3), *B. polybranchia* and *Ps. dayii* have been recorded on wild abalone along the South African coast (Simon, 2011; Boonzaaier *et al.* 2014).

Although records of polydorids infesting abalone in South Africa are extensive, relatively little is known about species infesting cultured oysters (*C. gigas*). Only three polydorid species have been recorded on farmed *C. gigas* on the east coast; *P. hoplura*, *Polydora* cf. *ciliata* and *D. keulderae* (Nel *et al*. 1996; Simon, 2011). A preliminary study conducted on five oyster farms in 2011, identified the most prevalent polydorids infesting *C. gigas* as *B. pseudonatrix*, *P. hoplura* and *P.* cf. *ciliata/calcarea* (de Lange *et al.* 2011). As a result, these species were selected for the purposes of this study.

Boccardia pseudonatrix Day, 1961 is endemic to South Africa and was first found boring into rock in the Knysna Estuary in South Africa (Day 1961). It has since been recorded on the eastern coast of the country at Haga Haga, Hamburg and Port Elizabeth (Simon *et al*. 2010; Simon and van Niekerk, 2012), and on oyster farms on the west coast of the country at Kleinzee and Paternoster (de Lange *et al.* 2011). *Boccardia pseudonatrix* was recently recorded in New Zealand where the speculated mode of introduction was ballast or ship hull-fouling (Glasby *et al.* 2009 in Cinar, 2013). It has also been recorded on *C. gigas* from New South Wales, and South Australia (Walker, 2014). Walker (2014) suggested that this species was most likely introduced to South Australia via New Zealand following ocean currents via fouled ship-hulls or natural and manmade objects. These represent the only records of this species outside its native range.

Polydora ciliata was first described as a tube dwelling, sand burrowing worm in Berwick, Scotland (Johnston, 1838). Since then *P. ciliata* has been recorded in Northern Europe, the Arctic, north-west Pacific, Australia and South Africa (Day, 1967; Walker, 2011), often as a shell-borer. Recently *Polydora* cf. *ciliata* was recorded on farmed oysters from Port Elizabeth and Hamburg (Simon, 2011). However, there has been some uncertainty regarding the identity of this species, since *Polydora ciliata* from southern Africa has never been found on molluscan shells, but rather found boring into calcareous rock and lithothamnion (Day, 1967). *Polydora ciliata*, which is a non-shell boring species, closely resembles the generalist shell-boring *Polydora calcarea* (Radashevsky and Pankova, 2006). As a result, specimens from the *P. ciliata* complex boring into lithothamnion and other calcareous materials (e.g. oyster shells) were referred to as *P.* cf. *calcarea* by de Lange *et al*. (2011). After the first record of this species by Simon (2011), it was found on oyster farms at Kleinzee and Swakopmund (de Lange *et al*. 2011). The increase in records of this species at oyster farms in recent years suggests that it may be anthropogenically dispersed between farms, possibly via the transport of oysters. Alternatively, it is possible that this species has always been present in the wild and researches have only recorded it in recent years. An accurate identification of *P. ciliata*/*calcarea* (using morphological and molecular data) is required since this may provide insight into the possible source of infestation of this species. In addition, the identification of this species is important since it is not known whether it is new to science, and whether it is potentially a recent introduction to southern African waters.

The type locality for *Polydora hoplura* is the Gulf of Naples, Italy (Claparéde, 1869). Its distribution range includes Europe, the North Atlantic from Ireland to the English Channel and has also been recorded in Australia, New Zealand and South Africa (Day, 1967; Read, 1975; Blake and Kudenov, 1978; Walker, 2011). *Polydora hoplura* is widely reported as a pest of aquaculture around the world, and has been recorded on cultured *C. gigas* in France (Ruellet, 2004 in Royer *et al.* 2006; Lejart and Hily, 2011), Australia (Blake and Kudenov, 1978; Lleonart *et al.* 2003; Walker, 2014) and New Zealand (Handley, 1995). However, *P. hoplura* closely resembles *P. uncinata* (Sato-Okoshi *et al.* 2008; Walker, 2014) and may in fact be the same species. If this is true, the distribution range of this species would be much greater as it would also include Japan and Chile (see Walker, 2011).

In South Africa, *P. hoplura* was first recorded in Saldanha Bay on the west coast in 1947 (Millard, 1952 in Mead *et al.* 2011a). Since then it has been recorded at more easterly locations along the south coast at Mossel Bay (Day, 1967), then further east at Port Elizabeth (Nel *et al.* 1996), and then at Haga Haga (Simon, 2011). More recently, de Lange *et al.* (2011) found it north of Saldanha Bay on the west coast at oyster farms in Paternoster and Kleinzee. It was also recorded at oyster farms in Saldanha Bay and Port Elizabeth in the same study (Figure 1.1).

Since the first records of *B. pseudonatrix* and *P. hoplura*, the known distribution of these species has increased (Figure 1.1). Similarly, since Simon (2011) first recorded *Polydora* cf. *calcarea*, it has been recorded at oyster farms along the west coast and in Namibia. This apparent range expansion may be the result of the movement of farmed oysters which can have serious implications for oyster farming in general. Alternatively, it may be a consequence of recent sampling efforts being more successful compared to previous attempts, and these animals may have already existed in their more recently recorded ranges. The species may have also dispersed among closely situated sites.

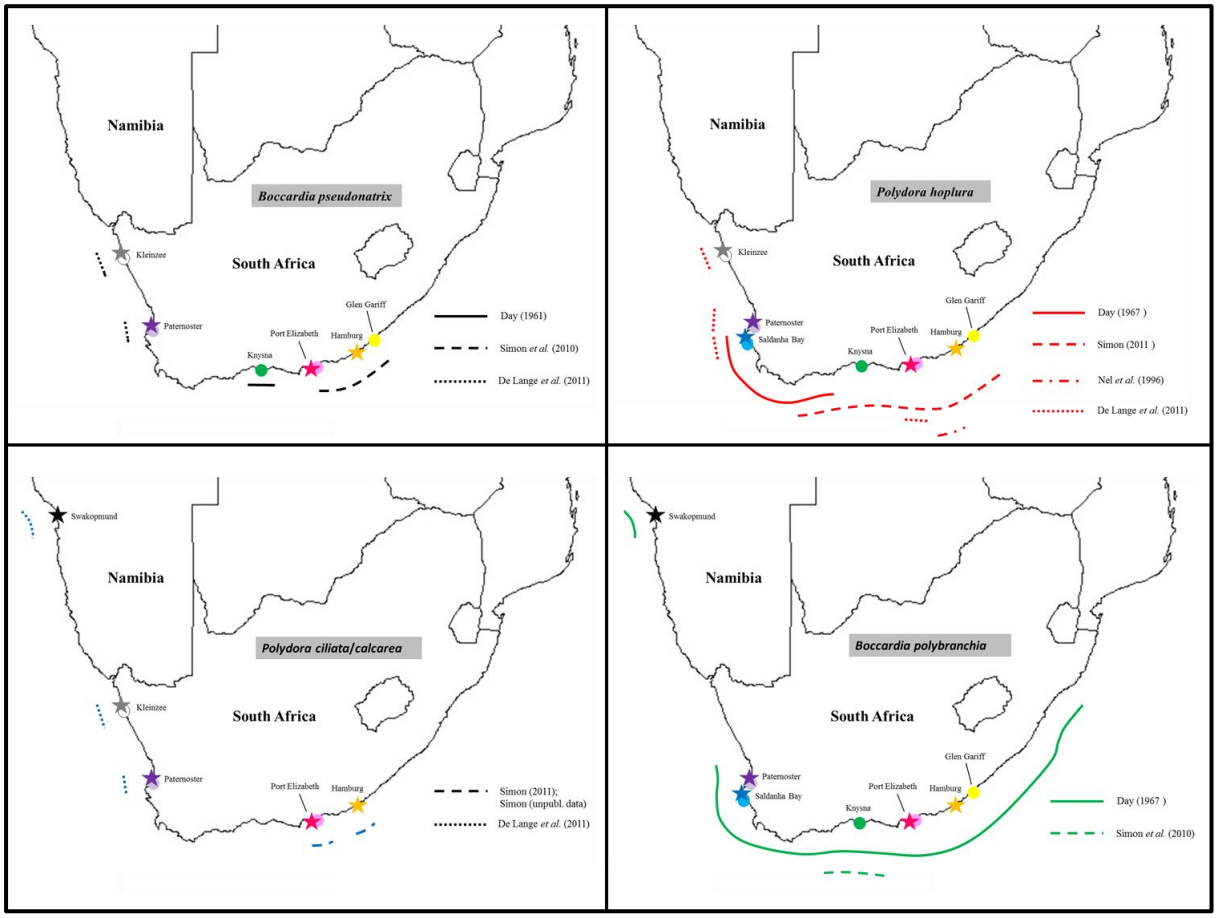

Figure 1.1 The distribution ranges of the three main oyster shell-infesting polydorids (*B. pseudonatrix*, *P. hoplura* and *Polydora ciliata*/*calcarea*) and *B. polybranchia* in southern Africa. Stars indicate the oyster farms relevant to this study and the circles are the wild sites.

Boccardia polybranchia is considered to have a cosmopolitan distribution and was first recorded on the rock oyster, *Saccostrea glomerulata* in New South Wales, Australia (Haswell, 1885). Since then it has been recorded in Chile, Brazil, Argentina, Peru, Tierra del Fuego, Straits of Magellan, Namibia, Biarritz, Gulf of Naples, Japan, English Channel, France, Iberian Peninsula and South Africa (Day, 1967; Walker, 2011). To tease apart the effects of the movement of pest polydorids with their hosts and their natural movement on population structure, it is advisable to use a reference/control species that a) is presumably not influenced by anthropogenic dispersal and b) has similar life history characteristics to the pest species making it comparable at the population genetic level. The perfect candidate is *Boccardia polybranchia* as it broadly meets the aforementioned requirements and has a large distribution range along the southern African coast (Augener, 1918; Day, 1967). In the present study I therefore used the population structure of *B.*

ploybranchia since the genetic distribution of this species is presumably only influenced by natural dispersal, which is most likely facilitated by the ocean currents (discussed later). Since no work has been done on the population structure of polydorid species along the SA coast, it is essential to determine the level of genetic connectivity in a naturally dispersing species. Similar patterns of genetic differentiation may be expected for the pest species if they too have dispersed naturally. Alternatively, anthropogenic dispersal can be inferred by the sharing of haplotypes among populations too distant for natural dispersal to have occurred (compared to the control species).

1. 5 Influence of larval development on dispersal

To better understand the dispersal capabilities of the study species, it is important to consider their reproductive strategies and life history patterns. Three larval developmental modes, presumably with differing dispersal capabilities, have been documented in polydorids; planktotrophy (with longer pelagic larval dispersal), and lecithotrophy and adelphophagy (with abbreviated larval dispersal). Some species may produce more than one type of larvae, and are known as "poecilogonous" (Blake and Arnofsky, 1999; Schulze, 2000). The species may produce planktotrophic and adelphophagic or lecithotrophic larvae, either in the same population or by the same individual (Blake and Arnofsky, 1999).

Species with planktotrophic larvae are highly fecund, and several thousands of larvae are released from the egg capsule at the 3-7 chaetiger (200-300 μm) stage. These larvae may remain pelagic for up to 85 days before they settle on a suitable substrate (Blake, 1969; Blake and Arnofsky, 1999). Lecithotrophic species are less fecund and larvae are provisioned with yolk from the female until they have developed 9-12 chaetigers (Blake, 1969; Blake and Arnofsky, 1999; Radashevsky and Nogueira, 2003). Adelphophagy is considered a variation of lecithotrophy since in both cases larvae are provided with yolk; in the former case, the yolk is in the form of nurse eggs which the larvae feed on, rather than development from large eggs that contain enough yolk for development in the latter (Blake and Arnofsky, 1999). These species generally have longer periods of brooding, where larvae survive off the yolk reserves until the reserves are exhausted. The advanced adelphophagic and lecithotrophic larvae leave the egg capsule and either spend a short time in the planktonic phase or settle directly on a suitable substrate.

Adelphophagy has been observed in *B. pseudonatrix* from the east (Haga Haga) and west (Kleinzee) coasts of South Africa (Simon, pers. obs.). The other species in this study are all peocilogonous. *Boccardia polybranchia* (Duchêne, 2000) and *P. ciliata*/*calcarea* produce both planktotrophic and adelphophagic larvae simultaneously (Simon, pers. obs.), whereas in *P. hoplura*, different individuals within populations produce offspring with different larval developmental modes (David *et al*. 2014). Poecilogony in the pest species may offer some reproductive advantages to proliferation, especially on onshore oyster farms (David *et al*. 2014).

In the marine environment longer pelagic larval duration is often associated with greater dispersal ability. This usually results in greater genetic connectivity among populations, often across large spatial scales (Palumbi, 1995). In contrast, abbreviated larval dispersal is usually associated with limited gene-flow and less genetic connectivity between populations, but is generally better suited for colonisation events (Palumbi, 1995; Bohonak, 1999; Cowen and Sponaugle, 2009). However, Teske *et al.* (2007a) determined that some marine invertebrate species (e.g. the crown crab, *Hymenosoma orbiculare*) with abbreviated larval development may have similar genetic structure as planktotrophic species along the southern African coast. It was hypothesised that these abbreviated developers maintain genetic connectivity via passive dispersal facilitated by ocean currents in the region. In the poecilogonous pest species, adelphophagic development may be crucial for the establishment and subsequent proliferation of local populations, from which range expansion can occur through planktotrophy (David *et al*. 2014). Furthermore, David *et al*. (2014) suggested that this reproductive flexibility offered by poecilogonous development may have aided the proliferation of some pest species on oyster farms in South Africa. Poecilogonous species may also be less susceptible to population bottlenecks, as planktotrophic individuals may disperse away from the source population, ultimately avoiding the depressive cost of inbreeding. In addition to this, the proliferation of the pest species may have been amplified in a farm setting because larvae have a reliable supply of food and substrates to settle on and lack predators that they may have encountered in the wild (David *et al.* 2014).

Importantly, once populations have become established in the wild, they may provide a source for re-infestation at farms, especially if they are able to inhabit ecologically diverse niches. In addition to this, the spread of these species along the coast is likely to be enhanced by oceanography in the region. Once introduced and/or established on farms, the larvae of the pest species may escape back into the wild. Moreover, factors such as temperature and nutrient

availability may have an effect on the rate of larval development and may therefore influence the dispersal of these species (Cowen and Sponaugle, 2009).

1.6 Oceanography and the influence of phylogeographic barriers on the distribution of genetic lineages of marine species in the region

The southern African coast is dominated by two major coastal currents: the warm southward flowing Agulhas Current bordering the east coast, and the cold northward flowing Benguela Current bordering the west coast (Branch and Branch, 1995). The south coast is characterised by the Agulhas ring eddies which move in a direction opposite to the Agulhas Current, located west from where the Agulhas Current deflects away from the Agulhas bank. The movement of eddies may result in the transport of small bodies of water (with associated organisms) that may become integrated with the Benguela Current system (Reason *et al*. 2006). The dynamic southern African coastal current system may have implications for genetic connectivity and dispersal of polydorid larvae, as pelagic larvae are likely to be dispersed following the direction of the ocean currents (Teske *et al.* 2007a; Muller *et al*. 2011). Gene flow estimates, however, have suggested that some species of fish are capable of utilising the Agulhas in-shore counter current (Figure 1.2) (Neethling *et al*. 2008; von der Heyden *et al*. 2008) and move eastwards. If ocean currents are the only means of transport, geographically close sites (e.g. Saldanha Bay and Paternoster, and Hamburg and Haga Haga) are expected to show a greater degree of genetic connectivity relative to sites that are more distant.

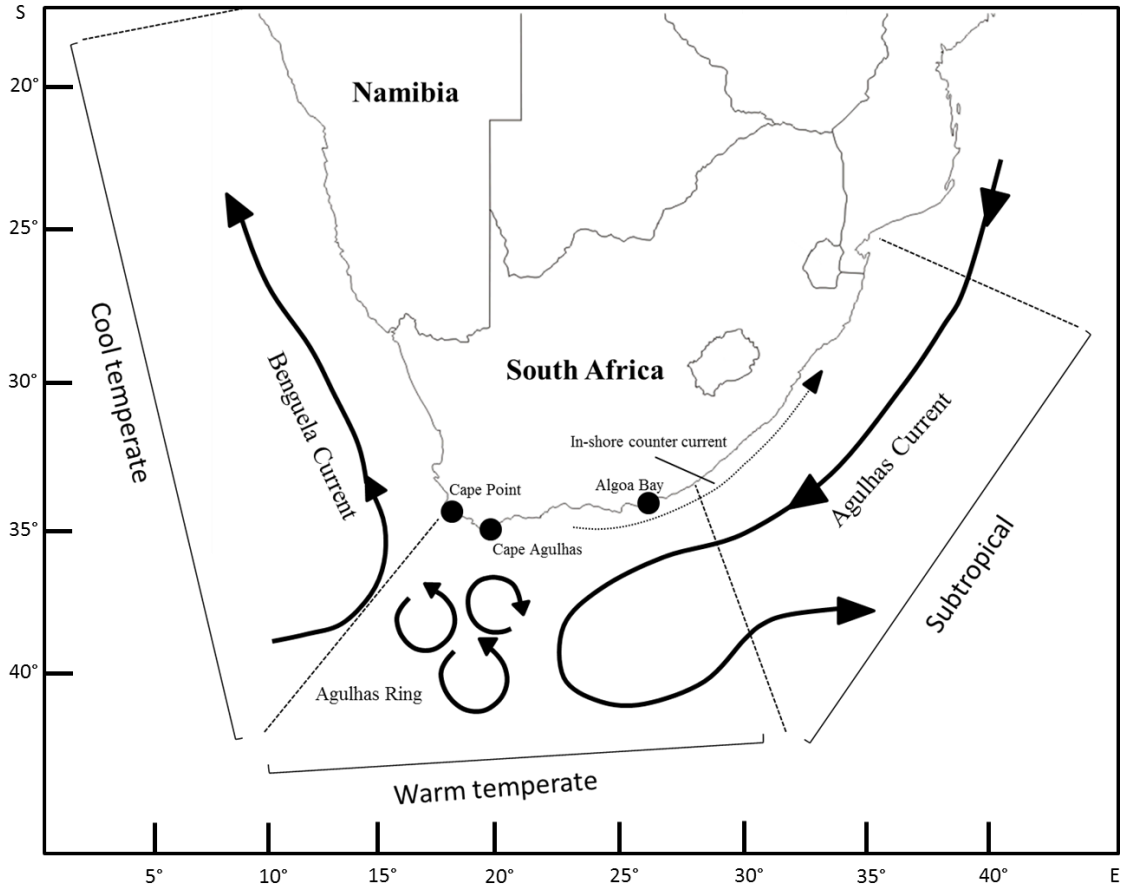

Figure 1.2. Map of southern Africa showing the major coastal currents. The warm Agulhas Current flows southward and the cold Benguela Current flows northward. The Agulhas ring eddies and in-shore counter current are also depicted (adapted from Harris, 1978 and Lutjeharms and Ansorge, 2001). The Cool temperate, Warm temperate and Subtropical biogeographic regions adapted from Sink *et al.* (2005) and Teske *et al.* (2011), as well as the documented vicariant phylogeographic breaks taken from Teske *et al*. (2011) are indicated along the coastline (Cape Point, Cape Agulhas and Algoa Bay).

The South African coast is characterised by five phylogeographic breaks that are found within four biogeographic regions (Teske *et al.* 2011). However, only three phylogeographic breaks and biogeographic regions are relevant to this study; Cape Point (separating the Cool temperate/warm temperate biogeographic regions), Cape Agulhas (centrally located in the Warm temperate biogeographic region) and Algoa Bay (separating the Warm temperate/Subtropical biogeographic regions) (Figure 1.2). The most prominent barriers to dispersal are at Cape Point and Cape Agulhas (Teske *et al*. 2011). The region between these barriers is considered a transition zone and several species (e.g. the isopod, *Exosphaeroma hylecoetes*; the mud prawn, *Upogebia*

africana; the cumacean, *Iphinoe truncata*; and abalone, *H midae*) have genetic lineages that are endemic to this transition zone (Teske *et al.* 2006, 2007a). Interestingly, Muller *et al.* (2011) found no genetic break in the Cape sea urchin (*Parechinus angulosus*) in the region of Cape Agulhas, but found strong genetic discontinuity at Cape Point. Gene-flow analysis revealed strong migration from east to west around Cape Point, but little to no migration from west to east (supporting the transport of small bodies of water from the Agulhas to the Benguela Current – see above). Similarly, Neethling *et al.* (2008) suggested that the planktonic larvae of the goby fish (*Caffrogobius caffer*) are also capable of traversing the Cape Agulhas barrier and in addition to this, it has been shown to utilise the inshore counter-current on the east coast. These studies highlight the complexity of dispersal patterns of species with planktonic larvae, across the transition zone at the Cape Point/Cape Agulhas region.

Along the east coast, phylogeographic breaks have been recognised at Port Alfred for the snail, *Nassarius krausianus,* possibly due to upwelling in the region (Teske *et al.* 2007b). Genetic discontinuity was also discovered for the cumacean, *I. truncata,* which was due to the Alexandria Coastal Dunefield. In the Algoa Bay region, isolated upwelling events are known to cause significant changes in water temperature, which in turn affects the distribution of marine species that are locally adapted to those conditions (Bolton, 1986; Schumann *et al*. 1988). Genetic disjunction has been found for two fishes in this region; *Clinus cottoides* (planktonic larvae) and *C. caffer* (larval developmental type not known). Populations of *C. caffer* generally showed no population structure based on mtDNA data, and gene-flow patterns revealed asymmetry; limited gene-flow from west to east, but strong gene-flow from east to west. This suggests a strong influence of the Agulhas Current on the direction of gene-flow in this species. However, there was limited gene-flow between Port Alfred and Haga Haga, the two furthest east populations, and more western populations, suggesting that populations are effectively isolated among these regions (Neethling *et al*. 2008). This was congruent with gene-flow patterns observed in *C. cottoides*, although populations of *C. cottoides* showed more genetic structure which coincides with the genetic breaks at Cape Agulhas and Cape Point (von der Heyden *et al*. 2008).

Overall, various studies have concluded that species with no planktonic phase are expected to be characterised by higher regional phylogeographic fragmentation, whereas planktotrophic species are expected to show higher levels of genetic connectivity. The existing oyster farms in South Africa are present in three biogeographical zones, spanning three phylogeographic breaks. The

Knysna oyster farm was the only farm located between the Cape Agulhas and Algoa Bay phylogeographic breaks. Since each of these biogeographic zones are generally characterised by distinct genetic assemblages (Teske *et al.* 2006; Teske *et al.* 2009), it is likely that natural dispersal of many taxa will be influenced by these. However, the distribution of genetic lineages in the pest species may depend on additional factors such as multiple introductions and anthropogenic movement.

Oyarzun *et al* (2011), working on the poecilogenous *B. proboscidea* in its native range on the West Coast of North America, showed that populations maintained genetic connectivity among sites in close proximity. However, a genetic break was realised at the California Transition Zone at Point Conception, a recognised biogeographic break for many marine species such as algae, barnacles and fishes (see references in Oyarzun *et al*. 2011). This suggests that even though poecilogonous species should presumably have dispersal advantages over species that have either planktotrophic or abbreviated larval development (see David *et al.* 2014), similar genetic patterns may be realised for all these developmental modes.

1.7 Aims of this study

Interpreting the genetic data of invasive species requires a thorough understanding of all the factors that may potentially influence genetic structure (Hastings *et al*. 2005). The differences among the oyster farming operations, vectors for dispersal (in this case the movement of *C. gigas* between farms), larval development and the oceanography in the region add multiple layers of complexity. The aims of this study were therefore to:

(1) Confirm the identity of *Polydora ciliata*/*calcarea*
(2) Determine the molecular structure and genetic diversity of the three pests and one reference control species to determine if the pest worms are
    a. anthropogenically moved with *C. gigas* between farms
    b. moving between farms and wild sites
    c. moving naturally, facilitated by ocean currents along the coast
(3) To determine whether the data gathered on the farming operations supports the genetic structure of the pest species

Chapter 2

Materials and Methods

2.1 Sample collection

Boccardia pseudonatrix, *Polydora hoplura* and *Polydora ciliata*/*calcarea* were collected from five oyster farms and six wild sites along the coast (Table 2.1). Thirty oysters were collected from each farm. Wild sites were sampled within 1 km from all farms except for Swakopmund and Hamburg where no individuals could be found in the wild. Various substrates that could potentially contain the study species were collected from wild sites (Table 2.2). These wild sites were selected to determine whether there is movement of larvae between farms and wild sites. I included Knysna as an additional wild site, due to the area's historical association with oyster farming (Hecht and Britz, 1990; Haupt *et al*. 2010a). Populations of the control species (*Boccardia polybranchia*) were collected from Paternoster, Saldanha Bay, Knysna and Port Elizabeth wild sites. I also sampled Glen Gariff (60 km's north of Hamburg farm) as Hamburg wild was devoid of suitable substrates that may have contained the pest polydorids (Steeman, pers. obs.). However, the only species recorded at this site was *B. polybranchia* which was included as an additional population to gain greater insight into the genetic distribution of this species. Specimens of the pest species collected as part of a morphological study by de Lange *et al*. (2011) were included in the present study. Additional specimens were provided by researchers mentioned in Table 2.1.

Table 2.1. Species sampled, sampling localities, sample sizes and collector of specimens. Notes: # indicates wild site. L. Williams (this study) is indicated by *, S.S. de Lange (2011) is indicated by + and C.A. Simon (2009) is indicated by ^.

Species	Locality/ (sample size)
Boccardia polybranchia	# Paternoster (14)* # Saldanha Bay (24)* # Knysna (26)* # Port Elizabeth (27)* # Glen Gariff (13)*
Boccardia pseudonatrix	Kleinzee (14)* + Paternoster (25)* + Hamburg (5)^
Polydora hoplura	Kleinzee (14)* + Paternoster (37)* + # Paternoster (14)* Saldanha Bay (39)* + # Saldanha Bay (24)* # Knysna (17)* Port Elizabeth (8)* # Port Elizabeth (15)*
Polydora ciliata/calcarea	Swakopmund (30)+ Kleinzee (16)* + Paternoster (2)* Hamburg (2)^

Table 2.2. Distribution of wild hosts/substrates sampled. Sites are listed from west to east. Abbreviations and notes: *Hm – Haliotis midae*, *Sm – Striostrea margaritacea*, *Ts – Turbo sarmaticus*, Ul– Unidentified limpet, *Hs – Haliclona* sp., Ca – Coralline algae, R – Rock; 1 – present, blank cell – not present at that site.

Wild sites	Distance from respective farm	Substrates						
		Hm	*Sm*	*Ts*	Ul	*Hs*	Ca	R
Kleinzee	0.5 km				1		1	1
Paternoster	10 km				1	1	1	1
Saldanha Bay	1 km				1	1	1	1
Knysna			1	1	1	1	1	1
Port Elizabeth	1 km		1		1	1	1	1
Glen Garrif		1		1	1	1	1	1

2.2 Sample processing

Molluscs were shucked and the shells and other substrates were placed in aerated seawater, on ice or stored at room temperature (maximum of 6 hours) before processing. Substrates were placed in a vermifuge (0.05% phenol in seawater) for two hours (Handley, 1995). This caused worms to leave their burrows; they were then removed and placed in petri dishes containing filtered seawater. Shells were then broken with cutting pliers to extract remaining worms. Individual worms were anaesthetised in 7% $MgCl_2$ in tap water (DeFelice *et al*. 2001) before identification on a LEICA L2 stereo-microscope and a LEICA DM1000 light-microscope. Polydorids were identified based on identification manuals and keys (Day, 1967; Simon and van Niekerk, 2012). Where possible, five individuals of each species per site were fixed in 4% formalin and stored in 70% ethanol to be used as voucher specimens. The remaining specimens were stored in 96% ethanol for molecular analyses.

2.3 Identification of *Polydora ciliata*/*calcarea*

2.3.1 **Morphological examination**

Polydora ciliata/*calcarea* collected from *Crassosrea gigas* from Kleinzee and Swakopmund farms in 2011 were used for morphological examination. Worms were extracted, preserved and identified as described above (sample processing) using identification keys and manuals (Blake, 1996; Read, 2010; Sato-Okoshi and Abe, 2013).

2.3.2 **Genetic techniques**

Total genomic DNA was extracted from members of the *Polydora ciliata*/*websteri* complex according to Blake (1996); *Polydora ciliata*/*calcarea* from Hamburg farm (this study), *Polydora websteri* and *Polydora neocaeca* from New York (provided by J.D. Williams, Hofstra University, New York, USA), *Polydora hoplura* from Saldanha Bay farm (this study), *Polydora* sp. from False Bay, South Africa (provided by C.A. Simon, Stellenbosch University, South Africa) using a Nucleospin® Tissue kit (Machery-Nagel, 2010) as specified by the manufacturer. Nuclear 18S rRNA sequences were amplified using the primers from Nishitani *et al.* (2012) (Table 2.3). The PCR cycling conditions were carried out based on Sato-Okoshi and Abe (2012a). Forward and reverse complementary sequences obtained from the three nucleotide sequences were combined

and aligned in Bioedit ver. 7.0.5.3. The combined sequences were authenticated using BLASTn (http://blast.ncbi.nlm.nih.gov). Neighbor-joining trees were generated in the program Molecular Evolutionary Genetics Analysis (MEGA) ver. 6.06 (Tamura *et al*. 2007) with default settings. Nodal support was assessed with 1000 bootstrap replicates (Felsenstein, 1985). Additional 18S rRNA sequences for members of the *Polydora ciliata*/*websteri* complex were obtained from GenBank (Species and accession numbers indicated in Figure 3.1, results section). *Polydora uncinata* is not listed as a member of the *P. ciliata*/*websteri* complex; however, it morphologically closely resembles *P. hoplura* (Sato-Okoshi *et al.* 2008), and sequences for this species were therefore included in the analysis. In addition to this, sequences for *Pygospio elegans* and *Marenzellaria viridus* were also obtained from GenBank, and were included as outgroup taxa based on Sato-Okoshi and Abe (2012).

2.4 Population genetics study

2.4.1 Molecular basis for study

Genetic markers can be especially useful when attempting to resolve invasion histories and can be suggestive of natural or anthropogenic dispersal (Roman, 2006; Darling *et al*. 2008). The rapid evolution of mitochondrial DNA in most species makes it a preferred marker of choice for population level analysis due to high nucleotide sequence variation between individuals (Avise, 1995). However, mtDNA is with a few exceptions strictly maternally inherited and is therefore not suitable where genetic hybridisation or male biased dispersal in concerned (Avise, 1995; Karl *et al.* 2012). For this reason, I used mtDNA and nuDNA genetic markers to determine the population structure of the study species.

The mtDNA Cytochome b marker has been used successfully to determine the level of genetic differentiation among populations of the polydorid *Boccardia proboscidea* (Simon *et al*. 2009; Oyarzun *et al*. 2011) and will therefore be used for the purposes of this study. Initially, I tested several nuclear markers; Internal transcribed spacer regions (ITS1 and 2): ITS1/ITS2 and ITS3/ITS4 (White *et al.* 1990); Lysidyl aminoacyl transfer RNA synthetase: LTRSf1/LTRSr1 and Adenine Nucleotide Transporter/ADP-ATP Translocase ANTf1/ANTr1 (Jarman *et al.* 2002). These attempts were all unsuccessful even after several PCR optimizations. However, after many optimisations (discussed later) the ATP synthetase subunit α (ATPsα) marker (Jarman *et al.*

2002) yielded positive results. This nuDNA marker was therefore used for the purposes of this study.

2.4.2 Molecular protocols

Total genomic DNA was extracted from whole worms using a Nucleospin® Tissue kit (Machery-Nagel, 2010) as specified by the manufacturer. An approximately 400 bp fragment of the mtDNA Cytochrome b gene was amplified and sequenced for all species (Table 2.3). An approximately 250 bp fragment of the ATPsα gene was initially amplified and sequenced using the forward and reverse primers from Jarman *et al*. (2002) (Table 2.3). This led to the amplification of more than one ATPsα allele for most of the individuals sequenced. Most of these sequences for *B. polybranchia*, *B. pseudonatrix* and *P. hoplura* displayed multiple-peak on the chromatogram, possibly due to a frame-shift caused by a deletion in one of the alleles. However, some consistent sequences could be read and these were used to design species specific reverse primers (Table 2.3). A new forward primer was also designed for *B. pseudonatrix* since there was difficulty amplifying using the original forward primer (Table 2.3).

Polymerase chain reactions were carried out using Super-Therm BioTaq DNA polymerase (JMR-801; Roche, Mannheim, Germany). Cycling parameters for the Cyt b gene were as follows: an initial denaturation of 5 min at 95˚C followed by 35 cycles of 30 secs denaturation at 95˚C, 30-60 secs annealing at 50˚C, and 30 secs extension at 72˚C, followed by a final extension period of 7 min at 72˚C. The same cycling parameters were applied when amplifying the ATPsα fragment, with the exception of the primer annealing temperature which ranged from 50 - 55 ˚C. All PCR products were separated by electrophoresis using a 1% agarose gel with ethidium bromide, for visual inspection under ultra-violet (UV) light. DNA amplicons of the expected size were excised and gel purified using a Bioflux® DNA/RNA extraction/purification kit (Bioer Technology Co., Ltd). The purified PCR products were re-suspended in elution buffer to adjust concentrations for effective sequencing. Purified PCR products were sequenced using BigDye chemistry and analysed using an Applied Biosystems 3730xl Genetic Analyser at the Central Analytical Facility (CAF) at Stellenbosch University.

Table 2.3 18S rRNA, Cyt b and species specific ATPsα primers used in this study.

Target gene	Target species	Primer	Sequence (5'-3')
18S rRNA	Members of the *P. ciliata*/*websteri* complex	18S-1F1[1]	AACCTGGTTGATYCTGCCAG
		18S-1R632[1]	ACTACGAGCTTTTTAACYGCARC
		18S-2F576[1]	GGTAATTCCAGCTCYAATRG
		18S-2R1209[1]	AAGTTTYCCCGTGTTGARTC
		18S-3F1129[1]	GCTGAAACTTAAAGRAATTGACGGA
		18S-3R1772[1]	TGGAGTGATTTGTCTGGTTRATTCCG
Cyt b	All species used for population genetic study	Cytb424F[2]	GGWTAYGTWYTWCCWTGRGGWCARAT
		Cytb-bp-876[3]	RAAWARRAAGTATCAYTCAGG
ATPsα	All species used for population genetic study	ATPsαF1[4]	GAGCCMATGCAGACTGGTATTAAGGCYGT
		ATPsαR1[4]	CTGTGGTAGTAGTTGGTCTTCKCNAAGTT
	B. polybranchia	BPolATPsαR[5]	GTCATCAAAGATCWTT
	P. hoplura	PHATPsαR1[5]	CATGAAAAAGGCACAATCCC
	B. pseudonatrix	BPsATPsαF1[5]	ATTGGCCGTGGTCAGCGTGA
	B. pseudonatrix	BPsATPsαR1[5]	CTTCTGGTTGATGATGGTGTC

[1]Nishitani *et al.* (2012); [2]Boore and Brown (2000) [3]Oyarzun *et al.* (2011) [4]Jarman *et al.* (2002); [5]Designed in this study.

2.4.3 Data analysis

Sequences were authenticated and aligned as described above. Clustal W2 online software (Larkin *et al.* 2007) was used to generate Phylogram trees to gain preliminary insight into the relationship among Cyt b and ATPsα sequences from individuals from different populations. Mitochondrial DNA sequences were translated to protein sequences using Expasy Translate (http://web.expasy.org/translate/) to confirm gene functionality. Identifying haplotypes from diploid organisms is often problematic since some individuals within a population may be heterozygous for multiple sites. The allelic phase of individuals heterozygous for the ATPsα gene region was therefore determined using the program PHASE in DnaSP ver. 5.10.01 (Librado and Rozas, 2009). The default settings were used; 100 iterations with a thinning interval of 1 and 100 burn-in generations.

To determine the evolutionary relationship among haplotypes, haplotype networks were generated for each species using TCS ver. 1.21 (Clement *et al*. 2000). Statistical confidence connections were generated at a 95% confidence limit. Genetic differentiation among populations was determined by calculating pairwise Φ_{ST} values for mtDNA and nuDNA data sets, and overall differentiations were calculated using an Analysis of Molecular Variance (AMOVA) in Arlequin ver. 3.1 (Excoffier *et al.* 2005). The default program settings were used to evaluate significance (0.05) at 1000 permutations. For *P. hoplura*, only two sequences were retrieved for the ATPsα gene region after various PCR optimisations. These sequences were not included when calculating the pair-wise Φ_{ST} values for this marker. Initial observations suggested that in populations of *B. polybranchia*, most of the variation was regionally confined. The data were therefore also split into *a priori* hierarchical regional eastern (Glen Gariff, Port Elizabeth and Knysna) and western (Saldanha Bay and Paternoster) clusters for the AMOVA analyses. Since *P. hoplura* is also widely distributed along the coast and also occurs in the three biogeographic regions which dominate the South African coast, sites were also split into eastern and western lineages to gain more insight into the possible effect of anthropogenic influence on its population structure. Haplotype (h) and nucleotide diversity (π) were calculated in DnaSP ver. 5.10.01 (Librado and Rozas, 2009).

2.4.4 Additional analysis for *Boccardia polybranchia*

To determine whether populations are isolated by distance, I performed an Isolation by distance (IBD) analysis using a Mantel test and 10 000 permutations in the program Alleles In Space (AIS) ver. 1.0 (Miller, 2005). To determine whether the known barriers to gene-flow could influence results obtained from the overarching IBD analysis (e.g. Teske *et al*. 2007a) populations from each biogeographic region was also analysed separately.

2.4.5 Questionnaire for farmers

A questionnaire consisting of 13 questions was designed to gather information that could supplement the molecular data. To gain insight into the possible origin of the pest species the following information was requested from the contributing farmers: (1) origin and (2) size of imported oyster spat/juveniles and, (3) whether the spat were in the sea in the country of origin? To establish whether the movement of *C. gigas* may have influenced the distribution of the pest species, the following information was requested; (4) are oysters (juvenile/mature or both) moved to other farms in southern Africa? Finally, to gain insight into whether it is possible for polychaete larvae to be exchanged between the farms and nearby wild substrates the following information was requested; (5) approximate volume of pond (if applicable);(6) distance between oyster farm and shore; (7) distance between neighbouring oyster nets/cages; (8) distance of oyster nets/cages from the seabed or pond bottom; (9) number of cohorts present on farm at any given moment; (10) is there a constant flow of water through pond area (if applicable), and approximate volume of flow through; (11) are there filters at the inflow, and if so, what size are the pores (micron); (12) Average time that oysters remain in the water column; (13) and the age of the farm?

Chapter 3

Results

3.1 Identification of *Polydora ciliata*/*calcarea* using morphological and genetic data

Polydora ciliata/*calcarea* closely resembles *P. websteri* from Japan and New Zealand (Read, 2010; Sato-Okoshi and Abe, 2013). Morphological similarities are listed below.

Dimensions: Up to 30 mm long for 100 segments.

Morphological characters: Prostomium is weakly incised, extending posteriorly as caruncle to the middle of chaetiger 3. Fine black line along feeding groove of palps, without banding. Four eyes are present in juveniles which may be lost in older individuals. Modified spines on chaetiger 5 are falcate with a lateral flange. Bidentate, hooded hooks begin on neuropodium of chaetiger 7, with 8-10 hooks per ramus. Branchiae begin on chaetiger 7 and continue up until posterior last ¼ of the body. Pygidium is disk-like with a wide dorsal notch.

Distribution and habitat: South Africa: Kleinzee, Hamburg; Namibia: Swakopmund (Simon, 2009; de Lange *et al.* 2011); possibly Mozambique as *P. ciliata* (Day, 1967). Recorded for the first time in Paternoster in 2012 (this study). All specimens from South Africa and Namibia were found on farmed *Crassostrea gigas*.

Molecular identification

Interspecific comparisons of the nuclear 18S rRNA sequences were based on 1759 bp. The phylogenetic position of all spionid taxa (Accession numbers included) are shown in Figure 3.1. Analysis of the 18S rRNA sequence data revealed that *P. ciliata*/*calcarea* differs by 2 bp (0.1%) from *P. websteri* from Japan and Australia. However, specimens from South Africa, Japan and Australia all differed markedly (29 bp or 1.6%) from *P. websteri* from the USA. The data in fact suggest a paraphyletic clustering for the two *P. websteri* lineages, suggesting that the species designation is in need of revision.

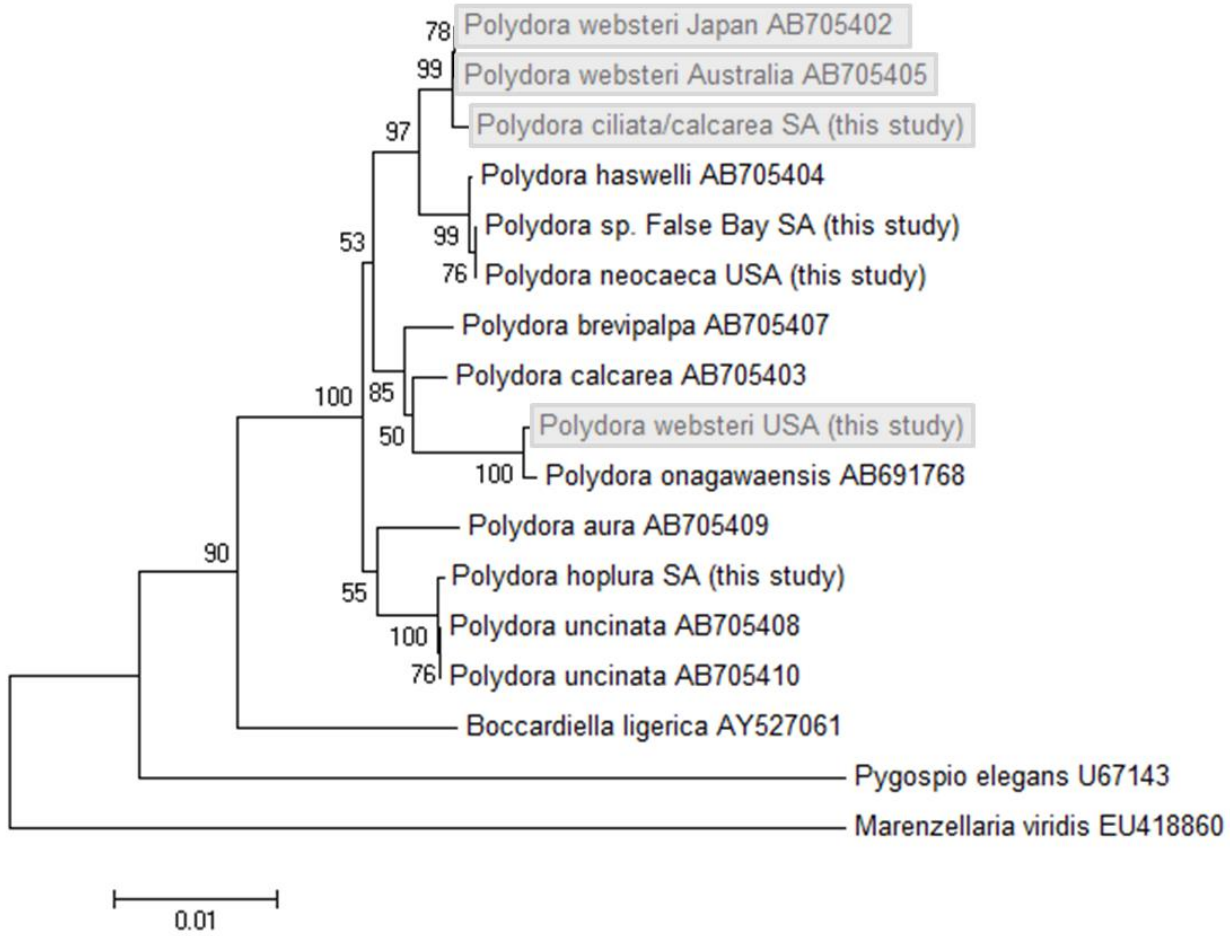

Figure 3.1. A neighbor-joining tree inferred from 18S rRNA sequences for members of the *Polydora ciliata*/*websteri* complex as defined by Blake (1996), *Boccardiella ligerica* and the spionids represented by *Pygospio elegans* and *Marenzelleria viridis*. Individuals that are highlighted were identified as *Polydora websteri* (*P. ciliata/calcarea* as *P. websteri* (this study); Sato-Okoshi and Abe, 2012; J.D. Williams, 2013). Bootstrap values greater than 50% are given at respective nodes. The scale bar represents the number of substitutions per site.

3.2 Population genetic study

3.2.1 Population structure of *Boccardia polybranchia*

The Cyt b dataset of 374 bp for 104 *Boccardia polybranchia* individuals revealed 11 variable positions. The ATPsα dataset of 179 bp for 46 sequences revealed 7 variable sites. Cytochrome b pairwise Φ_{ST} comparisons between eastern and western sites suggest a high level of divergence between sites from these regions, with no variation (negative Φ_{ST} values which were converted to zero) observed among sampling localities along the south and east costs. An intermediate level of divergence was presence among sampling localities along the west coast (Φ_{ST} = 0.153) (Table 3.1). Although individuals from the west coast were generally divergent from east coast

individuals, four individuals from the eastern clade (Knynsa) shared some haplotypes with individuals from the western clade. Divergence between eastern (Glen Gariff, Port Elizabeth and Knysna) and western (Saldanha Bay and Paternoster) clades is however very low (Table 3.1). The ATPsα data show significantly less divergence among western and eastern clades which was not congruent with the east/west divergence observed in the Cyt b dataset.

Nucleotide (π) and haplotype (h) diversity indices (Table 3.2) were higher for the west coast clade for Cyt b and ATPsα regions. TCS analyses of Cyt b and ATPsα data sets each yielded single haplotype networks connected at 95% confidence (Figure 3.2). The Cyt b network shows a high level of structure among eastern and western clade sites, whereas the ATPsα network generally shows a higher degree of haplotype sharing among these sites. A large number of individuals from the eastern lineage share the same Cyt b haplotype (n = 53); consisting of 13 individuals from Glen Gariff, 21 individuals from Port Elizabeth and 19 individuals from Knysna.

The ATPsα region is characterised by a high degree of haplotype sharing among eastern and western clades, whereas only four individuals from Knysna share a genetic signature with the west coast populations for Cyt b. This sharing of nuDNA haplotypes across the phylogeographic breaks at Cape Agulhas is probably reflective of ancestral polymorphisms present in the nuclear data. .

There was no significant relationship between mtDNA genetic and geographical distance, as determined by the Mantel test performed in AIS ($r = - 0.51$, $P > 0.05$). Analysing sites within each biogeographic region separately revealed that there is very low correlation between increased geographic and genetic distance among the south west coast sites ($r = 0.028$, $P > 0.05$). There was no correlation observed among the east coast sites ($r = -0.51$, $P > 0.05$).

Table 3.1. Pairwise Φ_{ST} values for Cytb and ATPsα datasets between the five *Boccardia polybranchia* populations sampled at wild sites along the south west, south and east coast of South Africa. W (in parenthesis) = Wild. Values above the diagonal are based on nuDNA data and values below the diagonal represent the mtDNA data. Bold indicates P < 0.05.

Locality	Glen Gariff (W)	Port Elizabeth (W)	Knysna (W)	Saldanha Bay (W)	Paternoster (W)
Glen Gariff (W)	-	0.000	0.000	0.000	0.109
Port Elizabeth (W)	0.000	-	0.000	0.000	0.000
Knysna (W)	0.000	0.000	-	0.000	0.093
Saldanha Bay (W)	**0.210**	**0.191**	**0.153**	-	0.021
Paternoster (W)	**0.268**	**0.251**	**0.192**	**0.153**	-

Table 3.2. Genetic diversity estimates for *Boccardia polybranchia*, using Cyt b and ATPsα sequence data. South eastern and south western sites are grouped. *N* refers to the number of individual sequences, *H* is the number of haplotypes retrieved, π is nucleotide diversity and *h* is haplotype diversity. Abbreviations: GG – Glen Gariff, PE – Port Elizabeth, Kn – Knysna, SB – Saldanha Bay, P – Paternoster

Coastal region	**Localities**	**mtDNA (Cyt b)**				**nuDNA (ATPsα)**			
		N	***H***	***π***	***h***	***N***	***H***	***π***	***h***
Eastern clade	GG, PE, Kn	66	7	0.001	0.350	32	7	0.010	0.659
Western clade	SB, P	38	6	0.006	0.654	14	8	0.014	0.934

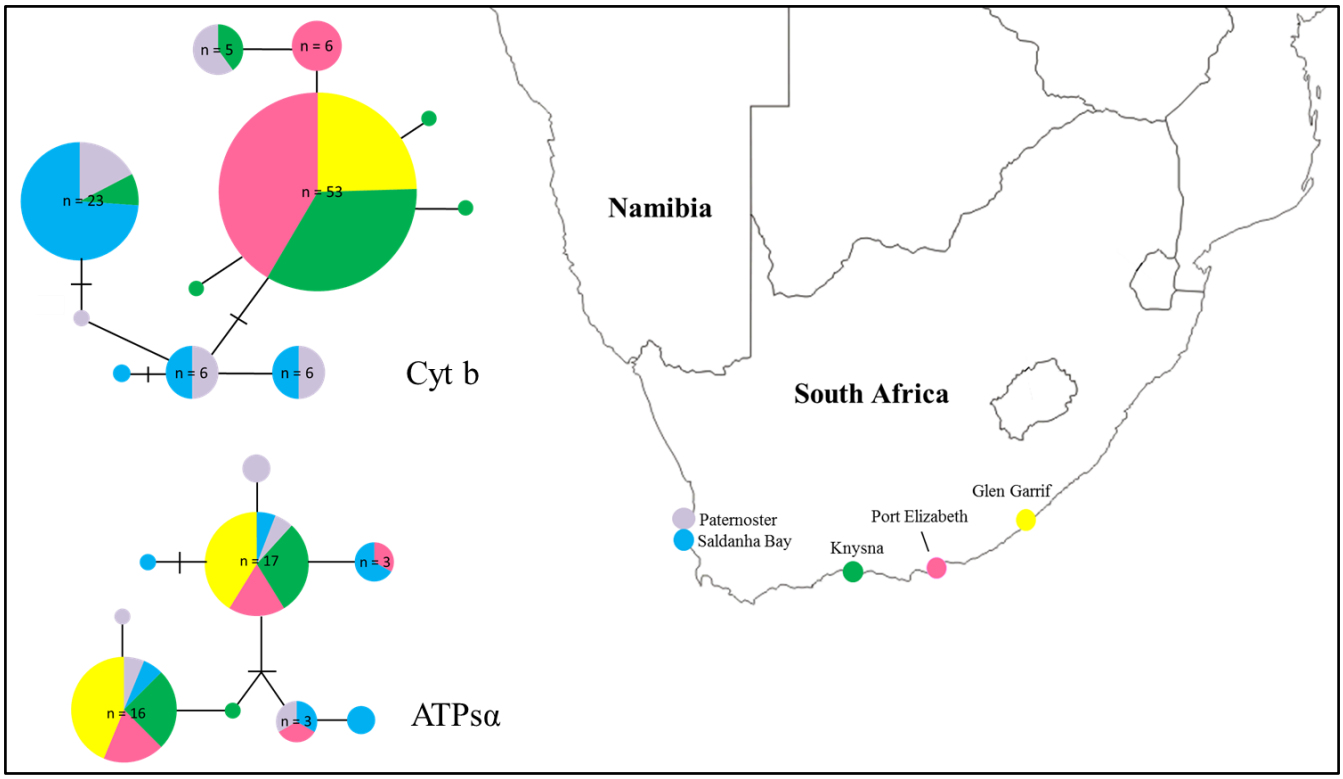

Figure 3.2 Cytochrome b and ATPsα haplotype networks for *B. polybranchia*. Each colour in the haplotype network corresponds to a specific site indicated on the map. Sites indicated as circles correspond to wild sites. The size of the circles in the haplotype network is proportional to the frequency of each haplotype; the smallest circles correspond to one haplotype. Lines connecting haplotypes indicate single mutational steps and lines perpendicular to these indicate additional mutational steps.

3.2.2 Population structure of *B. pseudonatrix*

The Cyt b dataset of 376 bp for 42 *Boccardia pseudonatrix* individuals revealed seven variable positions. The ATPsα dataset of 217 bp for 78 sequences revealed four variable sites. Pairwise Φ_{ST} values among sampling sites for Cyt b suggest a high level of differentiation between populations from Kleinzee and Paternoster farms (Φ_{ST} = 0.230; Table 3.3). The Cyt b TCS results suggest that populations from Paternoster and Hamburg are genetically more closely related when compared to Kleinzee and Paternoster (Figure 3.3). Pairwise Φ_{ST} values for ATPsα also suggests a significant level of divergence between populations from Paternoster and Kleinzee (Φ_{ST} = 0.140). For the ATPsα network, extensive haplotype sharing occurs across the three sites but interestingly, there

was one haplotype consisting of individuals from all three sites which could not be connected to the main network. A similar disconnected haplotype was not observed for the Cyt b network, and may be explained by the fact that this nuDNA genotype could have resulted from paternal sources coupled to ancestral polymorphisms (Avise, 2009). For the Cyt b data, the Kleinzee population showed the highest degree of nucleotide diversity (π = 0.041) and haplotype diversity (h = 0.571), whereas Hamburg was characterised by no nucleotide or haplotype diversity. A very low level of nucleotide diversity (π = 0.003 – 0.004) was obtained for all three populations for ATPsα, whereas haplotype diversity was relatively high (h = 0.629 – 0.668; Table 3.4).

Table 3.3. Pairwise ϕ_{ST} values for Cytb and ATPsα datasets between the three sampled populations. Values above the diagonal are based on nuDNA data and values below the diagonal represent the mtDNA data. F (in parentheses) = farm. Bold indicates $P < 0.05$. Negative values were converted to zero.

Locality	Kleinzee (F)	Paternoster (F)	Hamburg (F)
Kleinzee (F)	-	**0.140**	0.000
Paternoster (F)	**0.230**	-	0.098
Hamburg (F)	0.137	**0.143**	-

Table 3.4. Genetic diversity estimates for *B. pseudonatrix*, using Cyt b and ATPsα sequence data. Each sampling site was analysed separately for each marker. F (in parentheses) = farmed, *N* refers to the number of individual sequences, *H* is the number of haplotypes retrieved, π is nucleotide diversity and *h* is haplotype diversity.

Localities	**mtDNA (Cyt b)**				**nuDNA (ATPsα)**			
	N	***H***	***π***	***h***	***N***	***H***	***π***	***h***
Kleinzee (F)	14	4	0.041	0.571	34	4	0.004	0.668
Paternoster (F)	25	4	0.023	0.297	36	3	0.004	0.629
Hamburg (F)	5	1	0.000	0.000	8	4	0.003	0.643

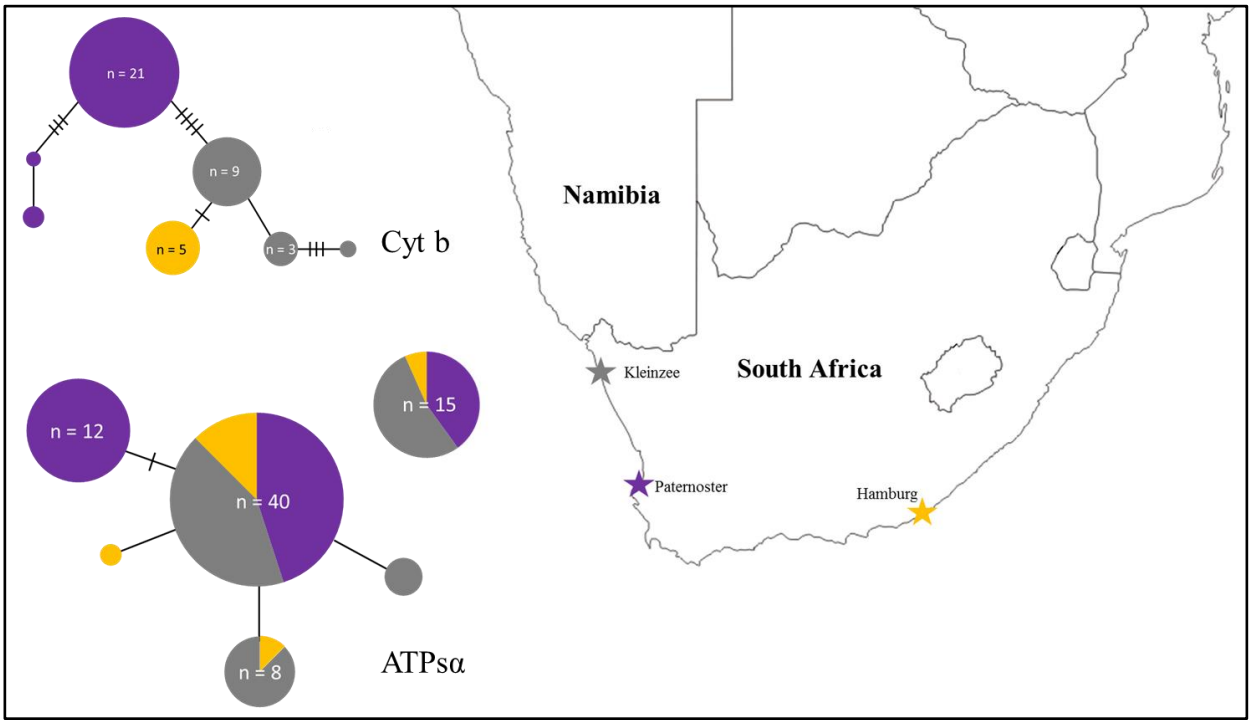

Figure 3.3 Cytochrome b and ATPsα haplotype networks for *Boccardia pseudonatrix*. Sampling sites (stars indicate farms) along the coast are shown.

3.2.3 Population structure of *P. hoplura*

The Cyt b dataset of 378 bp for 156 *Polydora hoplura* individuals consisted of 67 variable positions. The ATPsα dataset of 186 bp for 164 sequences consisted of 18 variable positions. Pairwise Φ_{ST} values for Cyt b (Table 3.5) indicate a low level of differentiation among some closely situated wild sites/offshore farms which are exposed to open ocean currents (e.g. Saldanha Bay farm/ Paternoster wild, Φ_{ST} = 0.012; Saldanha Bay wild/ Paternoster wild, Φ_{ST} = 0.007). There is a slightly higher level of differentiation among populations which are very far apart and separated by biogeographic barriers to dispersal (Cape Point and Cape Agulhas; e.g. Knysna wild/ Kleinzee farm, Φ_{ST} = 0.026; Port Elizabeth farm/ Saldanha Bay farm, Φ_{ST} = 0.027). However, this picture is not constant since there is a high level of differentiation among some sites which are geographically close together (e.g. Paternoster farm/wild, Φ_{ST} = 0.154; Paternoster farm/ Saldanha Bay wild, Φ_{ST} = 0.171). Overall, this indicates a mixture of genetic diversity across the geographic range and potentially points to anthropogenic movement among populations. Pairwise Φ_{ST} values for ATPs varied largely (Φ_{ST} = 0.016 – 0.670) among sites. High and low levels of divergence were observed for sites which are

closely situated (high divergence e.g. Paternoster farm/wild, Φ_{ST} = 0.668 and Paternoster farm/ Saldanha Bay farm, Φ_{ST} = 0.617; low divergence e.g. Paternoster farm/ Saldanha Bay wild, Φ_{ST} = 0.275; Knysna wild/ Port Elizabeth wild, Φ_{ST} = 0.041). There was also no evidence for genetic structure among sites which are far apart.

The TCS analysis revealed two separate haplotype networks for Cyt b and ATPsα datasets. None of the networks would connect after lowering the connection limit to 90%. As a result, connection limits were maintained at the 95% limit. All populations except Paternoster farm were generally characterised by high haplotype diversity for Cyt b (Table 3.6). The Cyt b haplotype network (Figure 3.4) and haplotype diversity (*h*) (Table 3.6) indicate a high level of genetic diversity in Saldanha Bay and Port Elizabeth farm and wild populations. High levels of genetic structure can be seen in the Cyt b haplotype data among neighbouring sites (Paternoster wild and Saldanha Bay farm and wild sites). There is however, sharing of Cyt b haplotypes among Kleinzee farm (n = 8 individuals) and Knysna (n = 10 individuals) across a large distance. There is also sharing among individuals from Paternoster wild and Port Elizabeth farm and wild sites, also across a large distance. The disconnected Cyt b haplotype consisting of 45 individuals with a larger central haplotype (n = 41) contained 40 Paternoster farm individuals, two Kleinzee farm individuals, two Knysna individuals and one Paternoster farm individual. Individuals from the n = 41 haplotype were genetically distinct from most other populations and consisted mainly of Paternoster farm individuals.

Table 3.5. Pairwise ϕ_{ST} values for Cytb and ATPsα datasets between the eight sampled *Polydora hoplura* populations. Values above the diagonal are based on nuDNA data and values below the diagonal represent the mtDNA data. Bold indicates $P < 0.05$.

Locality	Kleinzee (F)	Paternoster (F)	Paternoster (W)	Saldanha Bay (F)	Saldanha Bay (W)	Knysna (W)	Port Elizabeth (F)	Port Elizabeth (W)
Kleinzee (F)	-	**0.670**	**0.242**	**0.200**	**0.117**	0.044	-	0.016
Paternoster (F)	**0.192**	-	**0.668**	**0.617**	**0.275**	**0.591**	-	**0.356**
Paternoster (W)	**0.055**	**0.154**	-	**0.305**	**0.249**	**0.183**	-	**0.232**
Saldanha Bay (F)	**0.032**	**0.143**	0.012	-	**0.258**	**0.109**	-	**0.157**
Saldanha Bay (W)	**0.055**	**0.171**	0.007	**0.017**	-	**0.076**	-	0.036
Knysna (W)	0.026	**0.174**	**0.079**	**0.055**	**0.078**	-	-	0.041
Port Elizabeth (F)	0.061	**0.190**	0.043	0.027	**0.042**	**0.087**	-	-
Port Elizabeth (W)	**0.055**	**0.161**	**0.038**	**0.024**	**0.038**	**0.038**	0.042	-

Table 3.6. Genetic diversity statistics for *Polydora hoplura*, using Cyt b and ATPsα sequence data. Each sampling site was analysed separately for each marker. F and W (in parentheses) = farmed and wild respectively *N* refers to the number of individual sequences, *H* is the number of haplotypes retrieved, π is nucleotide diversity and *h* is haplotype diversity.

Localities	**mtDNA (Cyt b)**				**nuDNA (ATPsα)**			
	N	***H***	***π***	***h***	***N***	***H***	***π***	***h***
Kleinzee (F)	14	5	0.084	0.670	10	6	0.025	0.844
Paternoster (F)	37	4	0.005	0.158	30	1	0.000	0.000
Paternoster (W)	15	7	0.032	0.810	22	7	0.023	0.645
Saldanha Bay (F)	39	22	0.009	0.923	34	11	0.015	0.649
Saldanha Bay (W)	15	8	0.007	0.876	24	11	0.032	0.754
Knysna	17	7	0.024	0.765	12	9	0.025	0.955
Port Elizabeth (F)	8	6	0.135	0.893	-	-	-	-
Port Elizabeth (W)	15	8	0.007	0.867	26	7	0.026	0.766

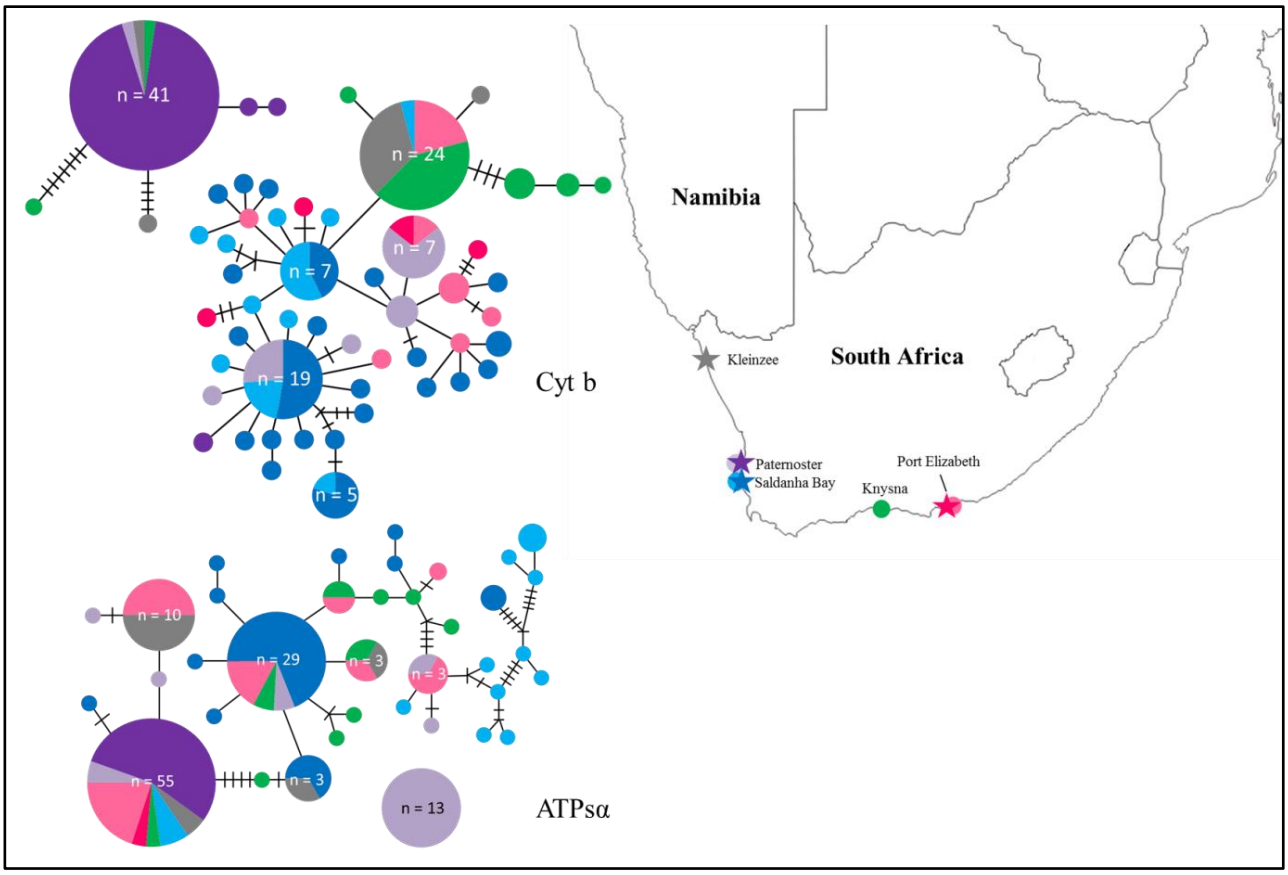

Fig. 3.4. Cytochrome b and ATPsα haplotype networks for *Polydora hoplura*. Sampling sites along the coast are indicated (circles indicate wild sites and stars indicate farms).

3.2.4 Population structure of *P. ciliata/calcarea*

The Cyt b dataset of 384 bp for the 50 *P. ciliata/calcarea* individuals showed no variation across all populations. The ATPsα dataset of 281 bp for 32 individuals revealed four variable sites. Populations of this species were characterised by remarkably low genetic diversity among the sampled localities, with the only observed variation found in the ATPsα data between the Hamburg population and Swakopmund, Kleinzee and Paternoster populations (Fig. 3.5).

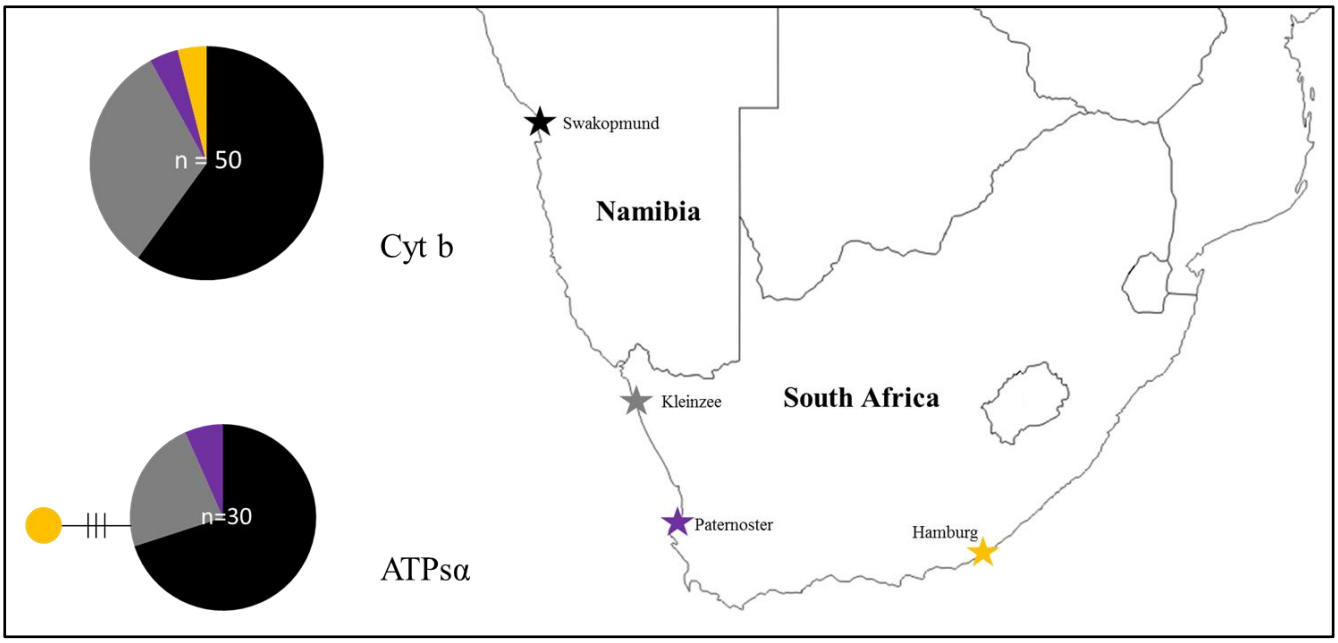

Fig. 3.5. Cytochrome b and ATPsα haplotype networks for *P. ciliata/calcarea*. Sampling sites (stars indicate farms) along the coast are indicated.

3.5 Questionnaire results

The oyster farms relevant to this study are characterised by some operational differences (Table 3.7) that may influence (1) the exposure of oysters to polydorid larvae, (2) the retention and dispersal of larvae and (3) the introduction and anthropogenic movement of worms among farms. Oysters at Swakopmund, Kleinzee and Paternoster are farmed onshore in ponds, while Saldanha Bay, Port Elizabeth and Hamburg farms are located offshore. The distances between neighbouring farms are given relative to the nearest west farm. The size of imported spat varied from 3-14 mm among sites however, the size of imported spat was confidential for Kleinzee farm. Distances of oyster cages from the floor ranged from 0.25-8 m among farms. All oyster farms are located within 0.1-1 km from the shore.

Table 3.7. Key differences among oyster farms relevant to this study. Abbreviations: BA(N) – Beira Aquaculture (Namibia), C(C) – Cultimar (Chile), CS(USA) – Coast Seafoods (USA), K(SA) – Kleinzee (South Africa). Farms are listed from west to east. Distances between neighboring farms are indicated as distance relative to nearest west farm

Farm	Distance relative to nearest west farm	Origin of spat 2010 to present	Size of imported spat	Distance of cages from floor	Onshore/ offshore farm	Flow rate of water	Distance from shore	Age of operation	Reference
Kleinzee		BA(N)	Confidential	Varies (No range given)	Onshore	Confidential	0.5 km	14 years	Quirin Snethlage Pers. Comm.
Paternoster	400 km	BA(N)	3 mm	0.5 m	Onshore	250 m^3/hour	0.1 km	1994-2012. Farm now closed	Kliffie Smit Pers. Comm.
Saldanha	40 km	K(SA)	14 mm	5 m	Offshore	Dependent on currents	1 km	4 years	Kevin Ruck Pers. Comm.
Port Elizabeth	820 km	BA(N), C(C), CS(USA), K(SA)	3-10 mm	8 m	Offshore	Depending on currents	1 km	15 years	Simon Burton Pers. Comm.
Hamburg	200 km	K(SA)	3 mm	0.25 m - 1.25 m	Offshore	Depending on currents	0.3 km	2004-2009. Re-opened 2012	Dave Krebser Pers. Comm.

Chapter 4

Discussion

4.1 Aims of this study

The purposes of this study were to 1) confirm the identity of *Polydora ciliata/calcarea* 2) determine the molecular structure and genetic diversity of the three main pests of oyster culture and one reference control species to determine whether pest are a) moving naturally between farms and close by wild sites, b) anthropogenically dispersed or c) moving naturally between farms, facilitated by ocean currents along the coast.

4.2 Confirming the identity of *Polydora* cf. *websteri*

Polydora cf. *websteri* collected in this study shares morphological similarities with several species: *P. aggassizi*, *P. aggregata*, *P. brevipalpa*, *P. ciliata*, *P. curiosa*, *P. haswelli*, *P. limicola*, *P. neocaeca* and *P. websteri* (Blake, 1996; Williams and Radashevsky, 1999; Radashevsky and Pankova, 2006; Read, 2010; Sato-Okoshi and Abe, 2012; Sato-Okoshi and Abe, 2013), of which all are characterised by fifth chaetiger falcate spines with a lateral subdistal flange/tooth, as defined by Blake (1996). However, some of these species are morphologically almost indistinguishable; e.g. *P. ciliata*, *P. websteri* and *Polydora haswelli* Blake and Kudenov (Blake and Kudenov, 1978; Read, 2010), and *P. ciliata* and *P. calcarea* (Radashevsky and Pankova, 2006) and could therefore be easily misidentified, which may have important ecological implications. Of these species, *P.* cf. *websteri* from South Africa most closely resembles *P. websteri* from Japan and Australia (Sato-Okoshi and Abe, 2013) as these species share various morphologically defining traits such as falcate spines with lateral flange on chaetiger 5, disk-like pygidium with a wide dorsal notch and a fine black line of pigmentation along the feeding groove of palps (Blake, 1996; Read, 2010; Sato-Okoshi and Abe, 2013). In addition, the molecular 18S rRNA data suggest that these taxa are synonymous and they only differ by 2 bp. However, *P. websteri* from the northeast coast of the USA differed markedly (29 site changes) suggesting that these species are different. Since American samples used in this study came from near the type locality of *P. websteri*, morphologically similar specimens from Japan, Australia and South Africa represent a genetically distinct species. Specimens from Japan and Australia should therefore also be referred to as *P.* cf. *websteri* as the description of this novel species was beyond the scope of this study.

P. cf. *websteri* from Japan and Australia are often associated with the formation of mud-blisters (Wisely *et al.* 1979; Sato-Okoshi and Abe, 2013; Walker, 2014), and similar observations have been made for specimens recorded on *Crassostrea gigas* in southern Africa. This, in addition to the morphological and molecular findings suggests that Japan or Australia may be the source of infestation in South Africa. However, "*P. websteri*" has also been recorded as a pest of various molluscan species in the USA (Loosanoff and Engle, 1943; Blake, 1996) and *C. gigas* from Hawaii (Bailey-Brock and Ringwood, 1982) and New Zealand (Handley and Bergquist, 1997). It is however uncertain whether the species responsible for these infestations are *P. websteri*, *P.* cf. *websteri* or both species. In the absence of knowledge whether both forms occur along the USA coastline, it is not possible to conclusively state the exact origin of the South African worms at this stage.

Although it remains unclear from where and how *P.* cf. *websteri* was introduced, it is likely that the secondary spread of this species occurred via the movement of *C. gigas*, since there is a single shared haplotype on all the farms where the species was recorded (i.e. Beira Aquaculture supplies Kleinzee, which supplies Saldanha Bay and Hamburg farms). Alternatively, but less parsimonious, three introductions occurred from the same source population.

4.3 Population genetic study

Among all the study species the mitochondrial marker was more sensitive in detecting differentiation and had greater resolving power in a phylogeographic context. The lack of phylogeographic resolution for ATPsα for the control species (*B. polybranchia*) and possibly the indigenous *B. pseudonatrix* (since there is little evidence for the anthropogenic dispersal of this species), is probably due to incomplete lineage sorting (Felsenstein, 2004) and the larger effective population size of nuDNA relative to mtDNA (Ballard and Whitlock, 2004) coupled to the retention of ancestral polymorphisms (Maddison and Knowles, 2006). For the introduced *P. hoplura*, the effect of multiple introductions from genetically diverse source populations and/or movement of molluscs associated with this species may have further contributed to the mixture of nuDNA alleles (see Roman and Darling, 2007) in this species.

In contrast to this, a low level a genetic divergence was reflected in both the mtDNA and nuDNA datasets for *P.* cf. *websteri*. Cytochrome b was characterised by a single haplotype and the only

variation in ATPsα was seen in individuals from Hamburg, where all of those individuals differed from the larger haplotype by the same 4 bp change. Since the Cyt b data were more informative for *B. polybranchia*, *B. pseudonatrix* and *P. hoplura*, I will base most of the discussions for these species on conclusions drawn for this marker. The mtDNA and nuDNA datasets will be taken into account when discussing the population structure of *P.* cf. *websteri* since the same conclusions can easily be drawn based on both gene regions.

For the introduced species (*P. hoplura* and *P.* cf. *websteri*), it is important to consider that the same Cyt b genetic signature at different sites may suggest two things: 1) that dispersal (natural or anthropogenic) has occurred between two or more sites, or 2) that populations were introduced from the same source population(s) that was homogeneous in nature, reflecting the same genetic Cyt b signature. As a consequence, if worms have been introduced from the same source population at different sites, they will share the same haplotypes without any natural or human-mediated means of dispersal.

4.3.1 Population structure of *Boccardia polybranchia* as facilitated by natural dispersal

The overarching purpose of including *B. polybranchia* in this study was to use its population structure as a control to illustrate natural dispersal and then to compare this to the population structure of the pest species. This would provide some insight into 1) the extent to which the distribution of the pest species could have been influenced by human-mediated dispersal, 2) how much of the dispersal could potentially be natural (among neighbouring sites) facilitated by the ocean currents, and 3) whether the distribution of the pest species has been influenced by the major phylogeographic barriers occurring in the region.

Although *B. polybrachia* was previously recorded on various molluscan shells (South African turban shell, *Turbo sarmaticus*; mussel, *Perna perna*; abalone, *Haliotis midae*; and limpet, *Scutellastra longicosta*: Simon *et al*. 2010), it was not associated with any molluscs sampled in this study, or on cultured abalone in a recent study (Boonzaaier *et al.* 2014). As a result, this species has presumably not been anthropogenically moved with molluscs. Therefore, genetic similarity between east (Knysna) and west (Saldanha Bay and Paternoster) are more than likely facilitated by natural dispersal.

4.3.2 Population structure within a biogeographic context

Populations of *B. polybranchia* were collected within its previously recorded distribution range (Day, 1967) coinciding with three South African biogeographic regions: cool-temperate, warm-temperate and subtropical, each of which generally has its own assemblage of genetic lineages with different marine invertebrate species (Teske *et al.* 2006; 2009). It can thus be predicted that marine invertebrates not studied has a great potential to be divided into two or three distinct genetic lineages across the biogeographic regions (Evans *et al*. 2004; Teske *et al*. 2006, 2007a, 2009; Zardi *et al.* 2007). It is therefore not surprising that our results suggest that *B. polybranchia* is divided into at least two genetic lineages separated by Cape Point as a biogeographic barrier.

Papadopoulos and Teske (2014) suggested that thermal adaptation of two southern African coastal crabs (*H. orbiculare* and *H. longicrure*) limits genetic exchange between adjacent populations of the same species. Thermal adaptation, however, does not explain the divergence among eastern and western lineages of *B. polybranchia* since there is considerable overlap (sharing of genetic lineages) between populations from subtropical (Glen Gariff) and warm temperate (Port Elizabeth and Knysna) biogeographic regions, and also sharing of genetic lineages between Knysna and both sites from the cold temperate (Saldanha Bay and Paternoster) biogeographic regions. The addition of sampling sites further west of Knysna may have revealed a higher degree of genetic connectivity among these biogeographically separated sites and may have provided more insight into the effect of the regional biogeographic conditions and phylogeographic breaks on the genetic structure of *B. polybranchia*.

4.3.3 Western phylogeographic breaks

Several invertebrate species are characterised by genetic discontinuity at the Cape Point region (e.g. the caridean shrimp, *Palaemon peringueyi*; the cumacean, *Iphinoe truncata*) (Teske *et al.* 2007a, 2007b), while in others, the genetic discontinuity is associated with the break at Cape Agulhas (e.g. abalone, *Haliotis midae*; the mudprawn, *Upogebia Africana*; *E. hylecoetes*) (Evans *et al*. 2004; Teske *et al*. 2006, 2007a, 2009; Zardi *et al*. 2007). Furthermore, several strong upwelling cells along the west coast of Africa (Laudien *et al*. 2003) resulting in strong population differentiation in the sea urchin, *Parechinus angulosis* (Muller *et al*. 2011). Our mtDNA results

suggest genetic discontinuity in *B. polybranchia* at either Cape Point or Cape Agulhas, or possibly both regions. However, the fact that Knysna (site closest to the western lineage) shares some haplotypes (n = 4) with sites from the western lineage suggests that this barrier to dispersal may be weak for polydorids (for example also see Neethling *et al*. 2008). Since samples were not collected between these phylogeographic barriers, it is impossible to determine the influence of each barrier on the population structure of *B. polybranchia*. However, since the oyster farms are located on either side of the transition zone (i.e. none are found between Cape Point and Cape Agulhas), the genetic data gathered on the population structure of *B. polybranchia* are sufficient for comparison with the pest species.

4.3.4 Eastern phylogeographic breaks

On the east coast there are several recognised phylogeographic breaks, for various taxa over a very short distance in the region: 1) Port Alfred due to coastal upwelling, e.g. the snail, *Nassarius kraussianus* (Teske *et al*. 2007b); 2) Sundays River and Bokness probably as a result of the Alexandria Coast Dunefield occurring between these localities, e.g. the cumacean, *I. truncata* (Teske *et al.* 2006) and; 3) Algoa Bay due to isolated upwelling events, e.g. *P. angulosus* (Muller *et al.* 2011) and two fishes: *Caffrogobius caffer* and *Clinus cottoides* (Neethling *et al.* 2008; von der Heyden *et al.* 2008). In contrast to these studies, a high degree of genetic connectivity was realised for *B. polybranchia* among sites from the eastern clade which are separated by approximately 500 km and confirmed by the absence of IBD between sites in that region. High levels of genetic connectivity across these regions have also been realised in other marine invertebrate species with long lived planktonic larvae (*U. africana* and *P. perna*) and abbreviated larval development (*H. orbiculare*) (Teske *et al*. 2007a), where it was speculated that the latter species maintained connectivity via passive dispersal facilitated by ocean currents.

Initially, gene flow was calculated for *B. polybranchia* but it was difficult to generate reliable estimates and draw robust conclusions on the migration patterns of this species. This was due to a small sample size and no genetic variation for the Glen Gariff population, and also due to a very low level of genetic variation (only 2 haplotypes for 27 individuals) for the Port Elizabeth population. Overall, the distribution of Cyt b lineages along the coast (east coast sites and west

coast sites form predominantly different clusters) supports the notion that the genetic structure of this species has been influenced by natural processes.

4.4 Population structure of the indigenous *Boccardia pseudonatrix*

Boccardia pseudonatrix was the only indigenous pest species infesting farmed *C. gigas* in this study. It was recorded at Paternoster and Kleinzee farms for the first time in a preliminary study (de Lange *et al*. 2011), and was also found at those sites in this study. If we assume that *B. pseudonatrix* is entering farms from the wild, then the genetic data suggest that this species is entering the farms at Kleinzee and Paternoster (and possibly also Hamburg) independently since there was no shared haplotypes for the Cyt b lineages between sites, as is shown for the introduced species (discussed later). In addition, the Cyt b data also indicate a relatively high level of divergence between populations from Kleinzee and Paternoster (ϕ_{ST} = 0.230).

Boccardia pseudonatrix was previously only reported to occur at Knysna on the east coast (Day, 1961), and only in recent years has it been recorded on molluscs; cultured *H. midae* and wild *Saccostrea cucullata* from Haga Haga and Port Elizabeth (Simon *et al.* 2010; Simon and van Niekerk, 2012; Boonzaaier *et al.* 2014). Given the genetic data for the three farms, it is likely that *B. pseudonatrix* has always existed in the wild in Kleinzee and Paternoster but was not previously found there due to undersampling. This hypothesis could not, unfortunately, be tested molecularly, as *B. pseudonatrix* was not recorded in the wild outside the respective farms.

Only two site changes in the Cyt b haplotype network separate worms from Hamburg and most individuals from Kleinzee (n = 9 haplotype). Notably, the Cyt b haplotype network and divergence estimates suggests that worms from Hamburg are genetically closer to worms from Kleinzee (ϕ_{ST} = 0.137), than what worms from Paternoster are to Kleinzee (ϕ_{ST} = 0.230). These two populations are the furthest apart and may thus indicate that this species may have been anthropogenically moved. This may be because an unsampled haplotype from Kleinzee was introduced at Hamburg. Another less likely explanation could be that worms that had been moved from Kleinzee to Hamburg underwent two mutational changes at the Cyt b region, which subsequently became fixed in the Hamburg population.

Since the movement of abalone between farms west and east of Cape Agulhas has been stopped to prevent the mixture of genetic stocks (Marine Aquaculture Permit Conditions: Abalone Hatchery, 2012), abalone no longer exist as a continued vector for the translocation of polydorids between the aforementioned regions. By contrast, pests such as *B. pseudonatrix* may still be moved via oyster transfer, especially since Kleinzee supplies most of the local farms along the coast (Chapter 3) with juvenile oysters. The worms may be transferred as epibionts on the juvenile *C. gigas*, or in the packaging as was suggested for the transport of *Polydora nuchalis* to Hawaii from Mexico (Bailey-Brock, 1990). Additionally, after the farm in Paternoster closed down in 2012, the remaining oysters were transferred to Saldanha Bay where *B. pseudonatrix* has never been recorded before, and as a result the worm may become established there in the wild, and on farmed oysters. However, conditions at Paternoster and Saldanha Bay farms may be very different, and this may influence retention and dispersal of polydorid larvae. It is likely that larval retention may occur more readily at the onshore farm in Paternoster compared to the offshore farm at Saldanha Bay. Although the adelphophagic larvae of *B. pseudonatrix* may settle shortly after being released from the egg capsule, worms are more likely to disperse further from the natal population in the wild or on offshore relative to onshore farms, which are confined to a specific volume.

As previously suggested, the worm may already be present in the wild, but in such low numbers that it has not been recorded before. On the west coast, *B. pseudonatrix* has only been recorded on onshore farms, where water temperatures are expected to be relatively higher compared to offshore environment, especially during summer (Rao, 2007). Previous records of this species in Haga Haga, Hamburg, Port Elizabeth and Knysna (Day, 1961; Simon *et al*. 2010; Simon and van Niekerk, 2012) were all associated with the warmer waters of the Agulhas Current, suggesting that higher temperatures may enhance the reproduction of this species.

A comparison of population structure of *B. pseudonatrix* with the control species (*B. polybranchia*) is difficult since *B. pseudonatrix* was recorded over a larger distribution range compared to *B. polybranchia*. Furthermore, no isolation by distance was found for *B. polybranchia*, and a relatively high level of population connectivity was realised for this species, suggesting that poecilogony may have positive implications for dispersal for *B. polybranchia*. However, *B. polybranchia* was not recorded at Kleinzee and as a result we cannot compare population genetic connectivity among these species between Kleinzee and Paternoster on the

west coast. Divergence between populations of *B. pseudonatrix* from Kleinzee and Paternoster may be maintained by regional environmental conditions or lack of long distance dispersal capabilities, as was shown for *E. hylecoetes* along the southern African coast (Teske *et al*. 2006). Furthermore, Muller *et al.* (2011) found significant isolation by distance and genetic discontinuity among sites along the west coast of South Africa which was attributed to upwelling cells disrupting gene flow in the region.

Overall, these results suggest that *B. pseudonatrix* is primarily infesting farmed *C. gigas* from Kleinzee and Paternoster independently from the wild. These results also provide circumstantial evidence for some movement of *B. pseudonatrix* between Kleinzee and Hamburg farms, but this conclusion is not well supported. Additional specimens from Hamburg may have provided some valuable insight into the relationship of those worms and the population from Kleinzee, as some haplotype sharing may have been evident among these sites.

4.5 Population structure of the introduced pest *Polydora hoplura*

The population structure of *P. hoplura* suggests four outcomes that are difficult to untangle since they are often not mutually exclusive to each other: 1) for some sampling sites there is evidence of structure among populations, 2) for others there is evidence of anthropogenic movement between sites that are far apart, 3) there is fairly good evidence for genetic/larval exchange between some farms and their nearby wild sites, and 4) there is evidence of multiple introductions from different places, reflected in the disconnected haplogroup.

4.5.1 Localised dispersal and genetic connectivity in *P. hoplura*

Localised dispersal of larvae among relatively close sites may explain some of the genetic connectivity seen among populations of *P. hoplura*, which has most likely occurred since this species was introduced. Records suggest that *P. hoplura* has been in South Africa since at least 1947 (Millard, 1952 in Mead *et al*. 2011a), although it may have been introduced hundreds of years ago (Chapter 1: historical and contemporary vectors of dispersal). The introduction of *P. hoplura* precedes the establishment of oyster farms in the region, so initial range expansion/s in this species would have been natural (depending whether it was a single or multiple

introductions), as occurred for the introduced mussel *Mytilus galloprovincialis.* This species was first detected in Saldanha Bay in the mid 1970's and spread naturally in a northerly direction along the west coast all the way to central Namibia, and eastwards along the south coast, but at a significantly slower rate (Branch and Steffani, 2004 and references therein).

Haplotype sharing between Saldanha Bay farm (n = 10) and wild (n = 4) and Paternoster wild sites (n = 5) suggests such localised dispersal of larvae, as was suggested for *B. polybranchia*. These sites are relatively close (approximately 40 km apart) and are not separated by known barriers to dispersal and as a result, natural dispersal is the most likely explanation for a shared mtDNA found between these sites. It is possible that localised dispersal of larvae may have occurred on the east coast, since there is haplotype sharing among Knysna (n = 10) and Port Elizabeth (n = 5). However, this is difficult to confirm and genetic connectivity between these sites may be due to a combination of both natural dispersal and anthropogenic dispersal, since regular movement of oysters has been documented between these sites (Haupt *et al.* 2012). Natural dispersal between Knysna and Port Elizabeth was evident for *B. polybranchia*, and has also been shown for other species such as the mussel *P. perna* (Nicastro *et al.* 2008) and *H. orbiculare* (Teske *et al.* 2007a); although in the former study individuals from Plettenberg Bay (approximately 30 km north east of Knysna) were used for genetic comparison.

4.5.2 Anthropogenic dispersal of *P. hoplura*

Although some species can disperse naturally over long distances (as expected for the poecilogonous *P. hoplura*), the existence of such dispersal mechanisms does not mean that the species was distributed naturally prior to human-mediated dispersal (Carlton, 1996). In this way human-mediated dispersal may result in distribution patterns which may be confused with natural dispersal. It is possible that genetic connectivity in *P. hoplura* between sites that are relatively close is as a result of human-mediated dispersal rather than natural dispersal. Regular movement of oysters between Knysna and Port Elizabeth has been documented, and it has been suggested that this movement most likely results in the translocation of fouling species such as *P. hoplura* among these sites (Haupt *et al*. 2012). Similar movement of oysters may have resulted in the movement of *P. hoplura* between farms (e.g. Kleinzee and Knysna; Paternoster and Port Elizabeth; possibly Port Elizabeth and Kleinzee).

The molecular data suggest the anthropogenic dispersal of *P. hoplura* between some sites, as was suggested for the spread of *Boccardia proboscidea* with the movement of abalone (Simon *et al*. 2009). However, this is only valid if we assume that the presence of the same Cyt b genetic signature at these sites is not as a result of separate introductions. A high level of haplotype sharing (n = 24 individuals) is evident among sites from eastern (Knysna = 10, Port Elizabeth = 5) and western (Saldanha Bay = 1, Kleinzee = 8) clades. As previously discussed, haplotype sharing between Knysna and Port Elizabeth wild may be as a result of human-mediated or natural dispersal, or a combination of both dispersal mechanisms. However, haplotype sharing among these east coast sites and Kleinzee farm which are far apart (approximately 1000 km from Knysna and approximately 1300 km from Port Elizabeth) and separated by the Cape Agulhas and Cape Point phylogeographic breaks is most likely more a result of human-mediated dispersal.

In *B. polybranchia*, individuals from eastern (Knysna) and western (Saldanha Bay and Paternoster) clades show some connectivity (via natural dispersal), but no connectivity was observed between Port Elizabeth and the western clades. This suggests that populations from Port Elizabeth are probably under stronger influence of the Agulhas Current (that deflects southwards) and cannot maintain connectivity via natural dispersal with sites on the west coast. In comparison, *P. hoplura* maintains some genetic connectivity between Port Elizabeth farm (n = 1) and wild (n = 1) and Paternoster wild (n = 5) providing some support that this species has been anthropogenically moved between these sites. As with *B. polybranchia*, *P. hoplura* shows some genetic connectivity between Knysna and Saldanha Bay which may be attributed either to natural or human-mediated dispersal. Alternatively, the presence of the same gene sequence at the four farms may be due to separate introductions from the same source and the presence of multiple genetic lineages at the same site may be as a result of many introductions from different source populations (Kolbe *et al*. 2004).

4.5.3 Genetic/larval exchange between farms and wild sites

The genetic data suggest larval dispersal between some farm and wild sites nearby. *Polydora hoplura* was recorded in the wild near oyster farms in Paternoster, Saldanha Bay and Port Elizabeth. The strongest evidence for dispersal between farm and wild sites was found in Saldanha Bay. Here the farm and wild sites were characterised by high mtDNA haplotype

diversity (farm h = 0.923, wild h = 0.876) but low nucleotide diversity (farm π = 0.009, wild π = 0.007) and show a strong degree of genetic structure, suggesting that the dispersal of worms occurs frequently between these sites. The high degree of genetic similarity observed between Saldanha Bay farm and wild populations strongly suggest that infestation at this farm is occurring mainly from nearby wild sources, as opposed to the introduction of worms from more distant sources.

At both Paternoster and Port Elizabeth farms, only one individual from the wild shares a haplotype with individuals from nearby farms, suggesting a relatively low degree of dispersal between these sites. At Paternoster farm 38 of 41 individuals were characterised by a single haplotype, suggesting that this is the dominant genetic signature for worms from the presumably very large population from there. As a result, the most likely explanation for genetic sharing is dispersal of worms from the farm into the wild. However, since a single individual from the wild shares this haplotype it is possibly that this Cyt b genotype entered the farm from the wild given that the distance between farm and wild is relatively short (0.1 km). Alternatively, the dominant haplotype for Paternoster farm individuals may be due to a recent introduction from a different source population, and genetic sharing (Cyt b) with Kleinzee and Knysna may then be explained by movement from Paternoster farm to those sites. The Port Elizabeth oyster farm is located approximately 1 km from the shore, which may influence larval exchange between the farm and the wild farm. In addition to this, oysters are grown in the open ocean in Port Elizabeth where the wild larvae may be carried away from where the oysters are grown.

Moreno *et al.* (2006) found evidence that some introduced pest polychaete species (e.g. *Dipolydora giardi* and *Polydora rickettsi*) were able to disperse locally to nearby wild populations of host species, following their introduction into Chile. Furthermore, it was suggested that this may have accelerated range expansion of these species within those regions. As such, it is likely that the infestation of wild molluscs led to the local establishment of these species, which in turn may have resulted in recurring infestations of molluscs by those species within that region. Therefore, since *P. hoplura* could have established centuries ago in South Africa, wild populations (which are widely distributed along the coast) are the most likely source of infestation at onshore farms. There is strong evidence suggesting this for populations from Saldanha Bay as there is a very high level of haplotype sharing among the farm and wild site. However, there may be constant exchange of genetic lineages among individuals from the farm

(via introductions) and wild site (established populations). Overall, this poses a significant problem for oyster farms in close proximity such as those in Saldanha Bay and Paternoster, since worms that may be introduced at either of these sites may easily disperse between these neighbouring wild sites.

4.5.4 Evidence for multiple introductions

It is often difficult to detect multiple introductions when individuals from the native range are not included for analysis (Kolbe *et al*. 2004). However, some factors suggest multiple introductions of *P. hoplura* into South Africa. Perhaps the most conspicuous is the disconnected haplogroup (n = 41) consisting of mostly Paternoster farm individuals, which suggests a separate introduction there from a genetically distinct population. The low level of divergence between Paternoster farm and the other sites (Φ_{ST} = 0.143 – 0.190), except Kleinzee, suggests that this is the same species and that the disconnecting haplotype is not as a result of the misidentification of individuals from Paternoster farm.

The Knysna Oyster Company was first established in 1948 (Hecht and Britz, 1990), and has since imported spat from France, Chile and the UK (Griffiths *et al.* 2009). However, since 2001, oyster production in Knysna declined to the point where it is no longer a significant producer. Instead, efforts have been concentrated on production in Saldanha Bay and Algoa Bay (Haupt *et al*. 2010a). Since oyster production at Knysna precedes production at all the other farms by at least 50 years, it may explain haplotype sharing of Knysna individuals with as many as five sites including Kleinzee and Paternoster farms and Paternoster, Saldanha Bay and Port Elizabeth wild sites. This suggests that Knysna is probably one of the source populations for infestation at the other sites.

Populations of *P. hoplura* from Port Elizabeth and Saldanha Bay, which are associated with international harbours, showed a high level of haplotype diversity. This is possibly due to multiple ship-borne introductions, since ballast water (Carlton and Gellar, 1993) and hull-fouling (Ruiz *et al*. 2000) are considered major vectors for the transport of polychaete larvae. Saldanha Bay populations show a low level of nucleotide diversity, which could indicate that these populations were introduced, possibly on multiple occasions, from a source within the native range of this species as individuals from these populations are expected to show a high level of

genetic connectivity. The high level of haplotype and nucleotide diversity for populations from Port Elizabeth suggests introductions from more than one source population. Such additive genetic variation is an expected genetic artefact of multiple introductions (Kolbe *et al.* 2004; Roman and Darling, 2007).

4.5.5 Distribution and possible origin of *P. hoplura*

The non-indigenous *P. hoplura* was the most widely recorded pest species along the South African coast. It was found on *C. gigas* from four of the five oyster farms sampled in this study, and was the only pest species recorded in the wild. *Polydora hoplura* was generally found within its previously recorded distribution range along the South African coast (Day, 1967), and was also found at Kleinzee farm for the first time in a preliminary study in 2011 (de Lange *et al.* 2011) and again in 2012. These were however, the first studies to sample Kleinzee farm for shell-infesting polydorids and the worm may have already been present at the farm since the farm was established in 2001.

Kleinzee nursery receives oyster spat from Beira Aquaculture (BA) in Swakopmund (Namibia). However, oysters from the neighbouring farm were only infested by *Polydora* cf. *websteri* suggesting that this may be an unlikely source of *P. hoplura* infestation at Kleinzee. Kleinzee oyster nursery primarily provides other farms with oyster spat for grow-out, and larger oysters are rarely moved to Kleinzee from other farms (Chapter 3). It is therefore unlikely that South African farms were the recent source of infestation at the nursery in Kleinzee. It was suggested that previous oyster imports from France had resulted in the introduction of the crab species *Xantho incisus* in Kleinzee (Haupt *et al*. 2010b). Such imports from regions within the native distribution of *P. hoplura* may have led to the introduction of the worm at Kleinzee, since the farm has only been operational since 2001.

The distribution of *P. hoplura* along the west coast (Chapter 1) suggests that this species is unable to traverse the gap between Paternoster and Kleinzee via natural dispersal. A reason for this may be the cold water upwelling along the west coast, which limits the mussel *Perna perna* distribution from the Cape of Good Hope to Lüderitz on the southern coast of Namibia (approximately 1000 km) (Zardi *et al.* 2007), or possibly the availability of suitable wild substrates along the west coast of South Africa.

Polydora hoplura showed no host specificity and was recorded on a wide variety of substrates in the wild including *S. margaritacea*, *T. surmaticus*, unidentified limpets, *Haliclona* sp. and coralline algae (Table 2.2). Most previous records of *P. hoplura* were from gastropod shells and other calcareous substrates (Day, 1967; Nel *et al*. 1996; Simon *et al.* 2006; Simon and Booth, 2007; Haupt *et al*. 2010a; Simon, 2011). Although no large gastropods were recorded in the wild on the west coast in this study, *P. hoplura* was found in *Haliclona* sp. and coralline algae at Paternoster and Saldanha Bay wild sites, of which both represent new records for substrates containing *P. hoplura* in South Africa. In addition to this, it should be noted that *Haliclona* sp. and coralline algae were the non-molluscan substrates on which the largest number of polydorid species were recorded: *B. proboscidea*, *Dipolydora capensis*, *P. hoplura*, *Polydora* sp., *Pseudopolydora* sp. (not reported in results). This is probably because these substrates were found in the greatest abundance along the coast, and large quantities could be processed from collections made during a single sampling event. Furthermore, *P. hoplura* was recorded in *Haliclona* sp. at all sites where present, and in coralline algae at most other sites. This suggests that the distribution *P. hoplura* along the southern African coast generally coincides with the distribution of these substrates, as opposed to the distribution of wild gastropods on which this species has been previously found.

Given that the native distribution range of *P. hoplura* includes European countries such as France, England and Portugal (Blake and Kudenov, 1978; Walker, 2011) from which the first shipping voyages to South Africa were made (Chapter 1), it seems reasonable to suggest that the worm may have been introduced from there hundreds of years ago. Additional introductions of this species may have occurred after the first imports of foreign oysters from England and France in the late 19th century (Hecht and Britz, 1990). However, populations within the native range of these species are required in order to confirm these speculations.

Polydora hoplura specimens from Australia were morphologically very similar to *Polydora uncinata* Sato-Okoshi (1988), and it was suggested that these species are synonymous (Walker, 2013). The 18S rRNA molecular phylogeny supports this, in that the specimen of *P. hoplura* from South Africa differs by only 1 bp from *P. uncinata* from Japan (Acc no: AB705408) and *P. uncinata* from Australia (Acc no: AB705410). Therefore, *P. hoplura* has very likely been spread over a much wider range than has been previously recorded (including Japan (Sato-Okoshi

(1998) and Chile (Radashevsky and Olivares (2005)). As a consequence Chile may have been an additional source of re-infestation and therefore genetic diversity for *P. hoplura* in South Africa.

4.6 Genetic structure of the introduced pest *Polydora* cf. *websteri*

All specimens collected across the four farms, with the greatest distance between sites exceeding 2000 km, are identical for Cyt b and show very low genetic diversity at the ATPsα region, strongly suggesting the anthropogenic movement and introduction from a single homogeneous source population. Sites on the east (Hamburg) and west (Paternoster, Kleinzee and Swakopmund) of southern Africa occur in two different biogeographic zones which are generally characterised by different genetic lineages of the same species (Teske *et al.* 2007a; Teske *et al.* 2011), which has also been shown for *B. polybranchia* in this study. In addition to this, east (Hamburg) and west coast sites (Paternoster and Kleinzee) are separated by three phylogeographic breaks (Cape Point, Cape Agulhas and Algoa Bay) for various taxa (Teske *et al.* 2011) including the control (*B. polybranchia*), providing additional support for the anthropogenic movement of this species.

The molecular Cyt b and ATPsα data suggest that *P.* cf. *websteri* may have been recently introduced from a source population with low genetic diversity (as seen in east coast populations of the control species). Similar observations have been made in other studies, for example, reduced genetic diversity in the mud snail *Batillaria attramentaria*, moved to North America with *C. gigas*, was most likely because successful transplants all came from a single prefecture in Japan (Miura *et al.* 2006). Alternatively, this species may have established from a small number of individuals, which may have been subject to the "founder effect" which results in the loss of genetic diversity (Roman and Darling, 2007). The 4 bp difference in the ATPsα data in individuals from Hamburg suggests that the worm may have been introduced there from a different source population. Alternatively, the presence of the same gene sequence at the four farms may be due to separate independent introductions from the same source (either from a small source population or region characterised by very low genetic diversity). However, the data on the movement of *C. gigas* in southern Africa (Chapter 3) confirms that there is movement of juvenile oysters between sites infested by this worm, making movement of oysters the more likely the cause for the spread of this shell-boring species.

The preliminary study by de Lange *et al.* (2011) represents the first records of this species in Swakopmund (Namibia) and Kleinzee on the west coast. Its distribution range was further extended in this study, when it was recorded in very low numbers (2 individuals) at Paternoster farm, suggesting that the worm was recently introduced there. This species was also recently recorded in high numbers (> 7 worms per shell) on oysters transported from Kleinzee to Hamburg, providing direct evidence for the anthropogenic movement of this species (pers. obs.).

4.7 The influence of oyster farm practices on the spread of the pest species (particular reference to *P. hoplura*)

Onshore farms would presumably be more prone to retention of larvae relative to offshore farms, where larvae may be more easily dispersed away from introduced source populations due to being openly exposed to ocean currents. As a result, a higher level of retention of previously introduced genetic lineages is expected for onshore farms. The extent of retention would depend on the age of the operation, source(s) of spat/mature oysters and flow through rate of water. However, information on the flow rate of water through respective farms was only given for Paternoster (250 m^3/hour), and as a consequence we cannot draw comparisons on how this may have influenced larval retention and therefore genetic structure. The population structure of the *P. hoplura* from Kleinzee suggests multiple introductions since the site is characterised by comparatively low haplotype diversity (h = 0.670) and high nucleotide diversity (π = 0.084). Kleinzee nursery currently receives spat from only Beira aquaculture (BA) however, it has previously received imports from Chile, France and the United Kingdom (Haupt *et al*. 2010a), which may have been the sources for genetic diversity observed for *P. hoplura*. A different scenario is seen for *P. hoplura* populations from Paternoster farm, where a dominant Cyt b lineage has become established. This farm also received spat from (BA) and there are no records of spat importation into Paternoster from international sources. This in combination with onshore farming conditions may have led to the proliferation and retention (David *et al*. 2014) of the dominant *P. hoplura* haplotype seen for most individuals from Paternoster farm, and may explain the low level of genetic diversity seen at this site.

However, as previously discussed, the contemporary population structure and genetic diversity in an introduced species (in this case *P. hoplura*) depends on the genetic diversity available in the

source population(s) (Roman and Darling, 2007). Given that specimens were only collected locally, it is difficult to determine whether the source populations for infestation in South Africa, also represent genetic lineages from introductions from other regions. If such introductions have occurred, the observed genetic diversity may represent fewer introductions or may possibly be as a result of a single introduction event at a specific site or in a specific region. Alternatively, low genetic structure in the source population will be reflected in the introduced population (Miura *et al.* 2006).

Oysters that are farmed in offshore environments are susceptible to the larvae of all fouling organisms that are present in the water column, and could therefore be infested by the full diversity of shell-boring species present in South Africa. More importantly, the genetic data effectively demonstrates how an introduced pest species may maintain genetic connectivity via natural dispersal among offshore farms and their surrounding wild sites (previously shown for Saldanha Bay farm and wild and Paternoster wild sites).

4.8 Possible steps to mitigate the spread of the pest species

Infestation by shell-boring polydorids may be inevitable, since even oyster farms that are isolated from the marine environment are subject to periodic infestation by species such as *P. websteri* (Bailey-Brock and Ringwood, 1982). Nel *et al.* (1996) determined that placing oysters in 70˚C seawater for 40 secs, or freshwater for 12 h significantly reduced, but not completely eradicated associated fouling organisms. Similarly, Haupt *et al.* (2012) showed that soaking oysters in fresh water for up to 18 hours significantly reduces the prevalence of *P. hoplura* (2.5% survival rate after 18 h). The heated seawater treatment completely eradicated *P. hoplura* after 60 secs of exposure, but 40 secs of exposure to the heated water is advised as a practical way to minimise oyster mortality. To further enhance eradication, Haupt *et al.* (2012) suggested that freshwater or heated seawater treatments should be carried out in addition to frequent inspections for fouling species before oysters are transported. Problem or invasive species are often only identified once they become problematic, and it is therefore essential to swiftly and accurately identify the species to prevent or control further spread. As a result, a sequence data-base to rapidly identify pest species (Karl, Rice and Simon, in review) is being developed. This will assist non-

polychaete specialists in the quick identification of these species which will be beneficial to the aquaculture industry.

4.9 Conclusion

This represents the first study on the population structure of polydorids in South Africa, and provides valuable information on how the movement of *C. gigas* may have influenced the dispersal of harmful shell-boring pests. The results strongly suggest anthropogenic movement for the non-indigenous *P. hoplura* and *P.* cf. *websteri* as these species show genetic connectivity over large distances often separated by more than one phylogeographic barrier. Furthermore, *P.* cf. *websteri* was recorded at Paternoster farm for the first time, but in very low numbers (n = 2) in 2012, suggesting a recent range expansion/introduction.

Boccardia polybranchia provided a useful blueprint for comparison, as its distribution was most likely not influenced anthropogenically, but was rather a consequence of only larval dispersal facilitated by ocean currents in the region. Larval development was not studied here although poecilogony has been reported for *B. polybranchia* from Kerguelen (Duchêne, 2000). However, Teske *et al.* (2007a) showed that marine invertebrates with planktonic and abbreviated developmental modes may be genetically similarly structured, since species may maintain genetic connectivity via passive dispersal mechanisms facilitated by the local ocean currents. Therefore, where dispersal is concerned, it may not matter if a species is capable of producing two different types of larvae. However, David *et al.* (2014) suggested that poecilogonous species may have the simultaneous advantage of forming robust natal populations from which planktotrophs can disperse from for range expansion.

Population structure does not always correlate to the life history of a species (Helmuth *et al.* 1994; Teske *et al.* 2007a; Ayre *et al.* 2009), and our results indicate connectivity across large distances which suggests an extended period of larval duration for South African specimens, a high level of genetic connectivity by passive dispersal facilitated by the ocean currents (Teske *et al*. 2007a). In contrast to this, genetic connectivity in populations of *B. pseudonatrix* could not be determined since populations of this species were very far apart, and no haplotype sharing was evident among populations. Although the effect of temperature on larval development was not studied here, the presence of this species only on onshore farms on the west coast (where water

temperatures are presumably higher than coastal water) and at many east coast sites associated with the warmer waters of the Agulhas Current, suggests that higher temperatures enhance successful reproduction in this species. However, additional research is required to confirm this speculation. Furthermore, there is some circumstantial evidence for the anthropogenic movement of *B. pseudonatrix* between Kleinzee and Hamburg.

The influence of oceanography and larval developmental mode on population genetic structure could only be evaluated in two of the study species: *B. polybranchia* and *P. hoplura*. The absence of *B. polybranchia* from all molluscs sampled in this study and the distribution of Cyt b lineages along the coast suggests natural dispersal facilitated by the ocean currents. For *P. hoplura* there is evidence for some localised dispersal among neighbouring populations (see earlier discussion). This localised dispersal of *P. hoplura* illustrates that introduced shell-boring pests may become established in the wild and may cause recurring problems of infestation for farmers if proper precautionary measures and monitory checks for pest species are not thorough. Consequently, it is possible that *P.* cf. *websteri* may also become established in the wild and may cause similar problems of re-infestation on nearby farmed oysters. Furthermore, future introductions of *P.* cf. *websteri* into South Africa from different source populations may lead to increased genetic diversity, which is likely to influence the invasiveness in this species.

Finally, the transport of molluscs is a notorious vector for the dispersal of harmful shell-boring species (e.g. Naylor *et al.* 2001; Wolff, 2005; Haupt *et al*. 2010a), and this study provides strong additional support for this hypothesis. This study also suggests that some invasive shell-boring polydorids may be successful invaders even when genetic diversity has been severely reduced (as seen for *P.* cf. *websteri*) and may be just as problematic as an introduced species with much higher genetic diversity (as seen for *P. hoplura*). In addition to this, the identification of introduced cryptic shell-boring pests, using morphological and molecular data is essential, since these species may be morphologically similar but reproductively very different which may influence invasiveness. Furthermore, accurately identifying these species may provide insight into the possible source of their introduction. Most importantly, these results suggest that caution should be exercised with the movement of molluscs since shell-boring polychaetes are moved with them.

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